THE NATURE OF THE BACTERICIDAL SUBSTANCE IN LEUCOCYTIC EXTRACT.*

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The phenomenon of phagocytosis furnishes evidence of the existence of powerful bacteriolytic substances within the cytoplasm of certain body cells. These endolysins are apparently quite distinct from serum bacteriolysins, since phagocytic cells are capable of destroying many bacteria that are not seriously injured by extracellular body fluids.

There is evidence that, under certain conditions, these intracellular lysins may be given off into the surrounding medium in sufficient quantities to play an important extracellular rôle. The automatic sterilization of old abscess cavities and the sterilization of the pneumonic lung could be accounted for by the assumption of the liberation of such endolysins as a result of cellular disintegration. This has given rise to the hope that eventually a valuable therapeutic agent may be obtained from phagocytic cells. It is conceivable that the therapeutic control of a number of infectious diseases, such as tuberculosis, pneumonia, and the various suppurations, may depend upon a knowledge of these substances. I have therefore undertaken to extend the present knowledge of these lysins, directing my initial effort to a determination of the approximate chemical nature of the bacteriolytic agent that can be extracted from leucocytes.

Considerable work has already been done with leucocytic extract.[1] Most investigators have studied rabbit leucocytes. These they have usually suspended in physiological saline, and have generally

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[1] For a bibliography and resumé of previous work on this subject, see Kling. Carl A., Untersuchungen über die bakterientötenden Eigenschaften der weissen Blutkörperchen, *Ztschr. f. Immunitätsforsch., Orig.*, 1910, vii, 1.

brought about the liberation of the bacteriolysin by repeated freezings and thawings, by heating the suspension to 50° C. for half an hour, or by simply allowing the suspension to undergo autolysis (table I).

TABLE I.

Bacteriolysin from Rabbit Leucocytes.

Twelve discarded rabbits were used as the source of the leucocytes. Each rabbit was injected in the right pleural cavity with 10 c.c. of a 5 per cent. suspension of aleuronat. The resulting pleural exudates were aspirated twenty-four hours later, added to an equal volume of 1.5 per cent. sodium citrate, and immediately centrifugalized. There were thus obtained about 10 c.c. of sediment consisting microscopically of a mixture of red and white cells in the approximate ratio of 1:1. No bacteria were seen, and samples transferred to agar gave no growth.

This sediment was washed twice by centrifugalization with physiological saline, was then suspended in four volumes of distilled water, and placed in the thermostat over night. In the morning the resulting extract was freed from cellular elements by centrifugalization, was heated to 58° C. for thirty minutes to destroy possible traces of serum bacteriolysin, and was then tested in various dilutions for its bactericidal power. In making these tests a loopful of an eighteen hour broth culture of *B. typhosus* was added to 1 c.c. of each fluid to be tested, and plates were made from the resulting mixtures at the times indicated. The table records the number of colonies on the plates thus obtained. The dilutions were made with fifth-physiological saline solution (p/5 sodium chlorid) to preserve osmotic relationships.

Material tested.	Time of plating.				
	1 min.	½ hr.	1¼ hrs.	3 hrs.	24 hrs.
Control, p/5 sodium chlorid	1,080	1,090	1,030	930	940
Undiluted extract.....	1	0	0	0	0
Diluted extract 1:2.....	20	0	0	0	3,000
Diluted extract 1:4.....	720	180	80	50	5,000
Diluted extract 1:8.....	1,240	1,100	1,200	1,340	1,000

The bactericidal agent thus obtained is relatively thermostable. It can be heated to 60° C. for an hour without loss of bactericidal power, and even to 80° C. without complete inactivation. This furnishes an easy means of differentiating this bacteriolysin from the serum bacteriolysin with which it may be mixed, since most sera are inactivated by heating to 55° C. for half an hour.

In addition to the thermostability, the most suggestive property of the extract for our present purposes is its loss of bactericidal

power on being passed through a Berkefeld filter (table II). From this it has been argued that the bacteriolysin is probably a colloid, presumably proteid in nature. Another suggestive property is the

TABLE II.

Effect of Filtration on Bacteriolysin.

Extract from rabbit leucocytes tested for its bactericidal power before and after its passage through a Berkefeld filter. Test organism, *B. typhosus.*

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4 hrs.	24 hrs.
Original extract.....	600	0	0	0
Filtered extract.....	1,120	1,020	950	20,000

apparent insolubility of the active principle in ether, which has been taken to indicate that the bacteriolysin is probably not a simple soap or lipoid. The extract can be evaporated to dryness without loss of its bactericidal properties.

The greatest handicap to a more extensive chemical study of the extract has arisen from the small amount of substance with which previous investigators have worked. By injecting aleuronat into the pleural cavity of a rabbit, one seldom obtains more than a single cubic centimeter of leucocytes, which on extraction yields but a few milligrams of dried bacteriolysins. It was thought that this handicap might be overcome by using as the source of material some large animal. The horse was therefore selected for this work.

Rabbit Leucocytes.—As an introduction to the study of horse leucocytes, most of the previous work with rabbit leucocytes was repeated, and the properties outlined above were confirmed. The knowledge of the nature of rabbit leucocytic extract was extended somewhat. Thus, it was shown that for the purpose of the experiment it was immaterial whether the leucocytes were obtained under septic or aseptic conditions. After heating the extracts to 60° C., the extracts obtained under septic conditions and those secured aseptically were apparently identical. Most of the septic extracts, however, if unheated, rapidly lost their bactericidal properties, due presumably to bacterial overgrowth.

The number of red blood corpuscles mixed with the leucocytes

was found to influence the nature of the extract obtained. In the earlier experiments the strongest and most uniform bacteriolysins were obtained from leucocytes mixed with a relatively large number of red cells. So uniform was this relationship, that it suggested the possibility of the bacteriolysin being a product of the red cells, rather than a direct product of the leucocytes. This, however, was shown not to be the case, since equally strong bacteriolysins were subsequently obtained from leucocytes free from red blood corpuscles. The presence of red cells, however, influences the autolytic processes leading to the liberation of the endolysin. The exact nature of this influence has not yet been determined.

In the earlier experiments it was noticed that the leucocytic extracts, freed from cellular elements, usually lost their bactericidal powers on standing. After four or five days in the ice chest, most of the earlier extracts were inactive. It was found that this deterioration could be prevented by heating the extract to 58° C. for thirty minutes. This heating presumably leads to a destruction of proteolytic enzymes responsible for the deterioration.

It was found that the extract could be freed from hemoglobin without loss of bactericidal power by heating it to 62.5° C. for twenty minutes. When so heated, most of the hemoglobin is coagulated, leaving the supernatant fluid nearly colorless.

In working with a substance whose presence or absence must be followed throughout a series of chemical manipulations by tests of bactericidal power, it is necessary to control the osmotic properties at each stage of the manipulation. An osmotic pressure much above that of physiological saline (0.85 per cent. sodium chlorid) is injurious to many bacteria. It was thought that the easiest way to control osmotic relations would be to free the initial extract as completely as possible from crystalloids. This was done by dialysis. It was found that the extract from the rabbit leucocytes could be dialyzed free from sodium chlorid, and presumably free from most of the other crystalloids, without loss of bacteriolytic properties. Celloidin sacs were used as the dialyzing membranes.

Horse Leucocytes.—With this introduction the study of horse leucocytes was begun. These were obtained by intrapleural injec-

tions of aleuronat. It was found advisable in making these injections to observe the following precautions.

1. The material injected should be sterile, and free from irritating substances. The presence of irritating or infectious agents usually causes a rapid pouring out of fluid into the pleural cavity, and extensive diapedesis of red blood cells, but causes the liberation of comparatively few leucocytes.

2. The amount injected should not be too large. Too large an injection causes the formation of a layer of fibrin and aleuronat over the pleural surfaces, through which layer leucocytes apparently have difficulty in making their way. From 300 to 500 cubic centimeters of 5 per cent. aleuronat in 2 per cent. starch paste is a sufficient dose, the starch being added to prevent too rapid sedimentation.

3. The injection should be made in such a manner as to distribute the aleuronat widely throughout the pleural cavity. I usually insert the needle close to the vertebral column, in order that part of the aleuronat may pass to the mediastinal surfaces.

4. The resulting exudate should be aspirated at frequent intervals, to prevent degeneration.

Following such an injection, there are usually formed from one to three liters of pleural exudate daily, for the first week, the daily yield then decreasing and usually ceasing about the fifteenth day. The exudate usually contains about 5 per cent. of leucocytes, often quite free from red blood corpuscles.

The extraction of a bactericidal substance from these leucocytes offers considerable difficulty. Most of the methods which gave successful extracts with rabbit leucocytes were tried, but the extracts obtained were almost uniformly without bactericidal power. One reason for this initial failure is the great variation, not only in the leucocytes of different horses, but also in the leucocytes of the same horse on different days after the aleuronat injection. Horse leucocytes are also apparently very easily injured by manipulation, and easily influenced in their autolytic processes. The exact nature of these factors I hope to make clear in a later paper.

Most of the earlier successful extracts were obtained from un-

washed leucocytes, mixed with comparatively large numbers of red blood corpuscles. This gave rise to the following method of extraction, used throughout this paper.

The pleural exudate, drawn into about a tenth of its volume of 3 per cent. sodium citrate, was enriched by the addition of a sufficient amount of freshly drawn citrated horse blood to make the ratio between the red and white cells approximately 1 to 1. The cells were then thrown down by centrifugalization, washed once at 0° C. (to prevent agglutination) with 50 per cent. horse serum, suspended in about four volumes of distilled water, incubated at 35° C. for three hours, and then packed in ice over night. The next morning the resulting extract was freed from cellular elements by centrifugalization, heated to 58° C. for thirty minutes, and tested for its bactericidal power (table III).

TABLE III.

Bactericidal Extracts from Horse Leucocytes.

Eleven extracts from horse leucocytes prepared by the technique described above. Test organism, *B. typhosus*.

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4½ hrs.	24 hrs.
Extract 1	1,520	1,280	1,120	5,000
Extract 2	1,100	880	730	750
Extract 3	980	760	280	550
Extract 4	750	75	10	60
Extract 5	1,000	190	35	0
Extract 6	400	6	2	0
Extract 7	930	128	0	0
Extract 8	580	2	0	0
Extract 9	600	0	0	0
Extract 10	120	0	0	0
Extract 11	4	0	0	0

About two thirds of the extracts prepared by this method were found to possess fairly strong bactericidal properties. The bactericidal power, however, was usually less than that of the extracts from rabbit leucocytes. It is probable that further study of the method of extraction may lead to stronger and more uniform results.

The bactericidal substance in these extracts has apparently the same general properties as the bactericidal substance from rabbit

leucocytes. There are minor differences, as in thermostability and in relation to dialysis, to be presented in detail in the final paper, but the agreement throughout is sufficiently close to warrant the belief that in this extract also the bactericidal agent is probably a proteid. The question then arises as to the class of proteid to which it belongs.

Proteids are usually divided into classes according to their solubility or insolubility in distilled water and in various salt solutions. The process of salting out, in which certain salts are added to a solution in certain concentrations, furnishes an easy means of separating proteids of different classes. This process was applied to the leucocytic extract, but with results that at first were both inconsistent and contradictory and markedly inconstant.

Thus, in one experiment ammonium sulphate was added to the extract to saturation. A heavy precipitate formed immediately. This was thrown down by centrifugalization, washed once with saturated ammonium sulphate, redissolved in a small volume of distilled water, and dialyzed free from ammonium sulphate. The solution thus obtained was evaporated to dryness *in vacuo*, and tested for its bactericidal properties. Making no allowance for amounts lost during manipulation, the end-product was found to possess approximately half the bactericidal power of the initial extract.

This experiment was then repeated, adopting precautions to make the determination an accurate quantitative one. A larger initial volume of extract was taken, the precipitate was dissolved in a larger amount of distilled water, the centrifuge tubes and pipettes were carefully rinsed out and the rinse water was added to the solution to be dialyzed. The end-product of this more careful determination was without bactericidal properties.

Here are two determinations, on superficial examination identical,—one giving a strongly bactericidal end-product, the other an end-product without bactericidal powers. This indicates either that the technique was greatly at fault, or that there are complexities in the determination not sufficiently taken into account in the experimental method.

The only apparent difference between the two experiments is a

volumetric one. As a result of the attempt to make the second determination a quantitative one, the second precipitate was dissolved in a larger volume of distilled water. It was thought that the resulting differences in concentration might possibly make a difference in the subsequent process of dialysis. A study was therefore made of the effect of dilution on dialysis, with the discovery that dilution alone is sufficient to destroy completely the bactericidal properties of the extract within the time necessary for dialysis (table IV).

TABLE IV.

Effect of Dilution on Bacteriolysin.

Sample of a concentrated extract from horse leucocytes diluted with fifty volumes of distilled water and allowed to stand in the ice chest for forty-eight hours. The fluid was then evaporated to dryness and tested for its bactericidal power. Test organism, *B. typhosus*.

Material tested.	Time of plating.			
	1 min.	2 hrs.	5 hrs.	24 hrs.
Diluted extract	920	720	700	1,200
Diluted extract 1: 2	940	820	740	1,100
Diluted extract 1: 4	930	750	770	1,200
Diluted extract 1: 8	900	720	730	800
Diluted extract 1:16	880	830	780	1,000
Original extract	820	110	3	0
Original extract 1: 2	800	30	3	0
Original extract 1: 4	700	40	1	0
Original extract 1: 8	890	610	330	24
Original extract 1:16	900	800	600	340

With this knowledge the experiment was repeated, the method being so modified as to avoid dilution. Instead of dissolving the precipitate in distilled water it was transferred to the dialyzing sac in the solid condition and allowed to go into solution in the small amount of water that entered the sac as a result of the differences in osmotic pressure. In this way it was found possible to dialyze the precipitate free from ammonium sulphate without allowing the volume at any time to increase above the initial volume from which the precipitate was obtained.

The products isolated by this method still show variations, but the results are sufficiently constant to warrant the provisional con-

clusion that the bactericidal substance is probably precipitated quantitatively by full saturation with ammonium sulphate (table V).

TABLE V.

Precipitation with Ammonium Sulphate.

To a sample of the salt-free extract pulverized ammonium sulphate was added to saturation, and the mixture was allowed to stand for one hour at room temperature. The resulting precipitate was thrown down by centrifugalization, washed once with a small amount of standard ammonium sulphate, and pressed between sheets of sterile filter paper to remove as much of the ammonium sulphate solution as possible. The solid precipitate was now transferred to a celloidin sac and dialyzed five times at 4° C. against 1,500 c.c. of double distilled water.

During the dialysis the precipitate went into solution in the small amount of water entering the sac, this volume increasing by the end of the dialysis to about two thirds of the original volume of the extract from which the precipitate was obtained. At the end of the dialysis this volume was made up to the original volume by the addition of distilled water, and the bactericidal power of the resulting solution was compared with that of the original extract. Test organism, *B. typhosus.*

Material tested.	Time of plating.			
	1 min.	2 hrs.	5 hrs.	24 hrs.
Precipitate	580	40	4	0
Precipitate 1: 2	580	20	3	0
Precipitate 1: 4	470	50	2	0
Precipitate 1: 8	1,020	1,120	720	20
Precipitate 1:16	1,010	940	810	550
Original extract	920	60	8	0
Original extract 1: 2	940	20	3	0
Original extract 1: 4	900	30	1	0
Original extract 1: 8	970	350	160	110
Original extract 1:16	1,040	870	770	810

The existence of antibactericidal substances in the extract not precipitated by ammonium sulphate may necessitate a subsequent modification of this conclusion. A study of precipitation by partial saturation with ammonium sulphate and precipitation with other salts is in progress, and will be reported in a later paper.

The earlier attempts to determine the relation of the bactericidal substance to alcoholic precipitation were also confusing. Samples of the extract were added to alcohols of various concentrations, the

resulting precipitates were thrown down by centrifugalization, and the supernatant alcohol decanted and evaporated to dryness. By this means the leucocytic extract was separated into two fractions,—an alcohol-soluble and an alcohol-insoluble part. These two fractions were taken up, either in distilled water or in physiological saline, or usually in some dilution of physiological saline, as fifth-physiological or tenth-physiological, and tested for their bactericidal properties.

In estimating bactericidal power it is desirable to have the osmotic pressures the same in the various parallel fluids to be tested. The dissolving and diluting fluids used were, therefore, those that would make the osmotic pressures equal throughout the determination. The results of these determinations were contradictory and uncertain.

Part of this early confusion I can now attribute to a destruction of the bacteriolysin as a result of dilution, a source of error not suspected at the time the determinations were made. A second source of error was subsequently discovered, the wholly unsuspected antibacteriolytic action of sodium chlorid.

It was early observed that the extracts from horse leucocytes usually increased considerably in bactericidal power on dialysis (table VI). This was at first attributed to the removal of inhibiting proteids as a result of the routine reheating at the end of dialysis, but a subsequent study showed that dialysis alone caused such an increase.

TABLE VI.

Effect of Dialysis on Bacteriolysin.

Samples of extract from horse leucocytes tested before and after dialysis. The dialyzed sample was reheated to insure sterility. Test organism, *B. typhosus.*

	Time of plating.			
	1 min.	1½ hrs.	4½ hrs.	24 hrs.
Before dialysis	780	560	330	900
After dialysis	550	180	8	0

Sodium chlorid added to a salt-free extract generally decreases its bactericidal power (table VII), the presence of 0.85 per cent.

sodium chlorid often being sufficient to inhibit completely bacteriolysis, while as little as 0.2 per cent. sodium chlorid produces an appreciable lessening. There was, therefore, sufficient sodium chlorid in most of the solvents employed in the earlier tests to render these determinations inconclusive. In repeating the work, distilled water was selected as the dissolving and diluting medium.

TABLE VII.

Effect of Sodium Chlorid on Bacteriolysin.

Samples of a salt-free purified bacteriolysin from horse leucocytes tested for its bactericidal power both alone and in the presence of increasing amounts of sodium chlorid. Test organism, *B. typhosus.*

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4½ hrs.	24 hrs.
Bacteriolysin, water	790	210	15	0
Bacteriolysin, +0.2 per cent. sodium chlorid	700	120	25	2,500
Bacteriolysin, +0.4 per cent. sodium chlorid	1,090	480	90	5,000
Bacteriolysin, +0.85 per cent. sodium chlorid	1,180	750	660	10,000

If leucocytic extract is added to absolute alcohol, a precipitate forms immediately. If this precipitate is washed with absolute ether and dried *in vacuo,* there is obtained a product soluble in distilled water, possessing about half the bactericidal power of the original extract. If, instead of immediately washing the precipitate with ether, it is washed with absolute alcohol, and is then dried, the final product is largely insoluble. By employing distilled water, however, a fairly strong bactericidal agent can be extracted from this precipitate. This water extract is free from hemoglobin and is presumably free from many other contaminating proteids. It represents the bactericidal agent in the highest degree of purity thus far obtained. The properties of this purified bacteriolysin are now under investigation.

No final conclusion can yet be drawn as to the nature of the bactericidal substance in leucocytic extract. Its behavior toward ammonium sulphate and alcohol are sufficient, however, to warrant the belief that the bactericidal substance is possibly an enzyme. Upon this supposition the investigation is being continued.

CONCLUSIONS.

1. The bactericidal agent extracted from horse leucocytes is apparently precipitated quantitatively by full saturation with ammonium sulphate.

2. The bactericidal agent is apparently precipitated by absolute alcohol, and is not rendered insoluble by a short contact with alcohol. The agent resembled in this feature certain enzymes which can be isolated and purified by alcoholic precipitation.

PURE CULTIVATION OF SPIROCHÆTA PHAGEDENIS (NEW SPECIES), A SPIRAL ORGANISM FOUND IN PHAGEDENIC LESIONS ON HUMAN EXTERNAL GENITALIA.*

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PLATES 32 AND 33.

In the ulcerative processes situated around the genital region of man one encounters, besides various forms of bacteria, a number of spiral microörganisms belonging to the genus Spirochæta or Treponema. Thus Schaudinn and Hoffmann[1] described *Spirochæta refringens;* von Prowazek and Hoffmann,[2] *Spirochæta balanitidis;* Róna,[3] *Spirochæta gangrænosa nosocomialis;* Corbus and Harris,[4] forms resembling Vincent's spirillum; Mulzer,[5] *Spirochæta pseudopallida;* and Polland,[6] a large spirochæta with five or six blunt curves.

While *Spirochæta refringens* is now known to be non-pathogenic, the etiological relation of *Spirochæta balanitidis* to balanitis erosiva circinata or that of *Spirochæta gangrænosa nosocomialis* to ulcus gangrænosum genitalicum has not been definitely established. Several difficulties must be overcome in order to settle this question.

First of all, it is not easy by morphology alone satisfactorily to identify a spiral organism found in certain lesions with the organisms previously described by these authorities. For example, there

* Received for publication, May 15, 1912.

[1] Schaudinn, F., and Hoffmann, E., *Arb. a. d. k. Gsndhtsamte,* 1905, xxii, 527; *Berl. klin. Wchnschr.,* 1905, xlii, 673.

[2] Hoffmann, E., and von Prowazek, S., *Centralbl. f. Bakteriol., Orig.,* 1906, xli, 741, 817.

[3] Róna, S., *Verhandl. d. deutsch. dermat. Gesellsch.,* 1907, ix, 471.

[4] Corbus, B. C., and Harris, F. G., *Jour. Am. Med. Assn.,* 1909, lii, 1474.

[5] Mulzer, P., *Berl. klin. Wchnschr.,* 1905, xlii, 1144.

[6] Polland, R., *Wien. klin. Wchnschr.,* 1905, xviii, 1236.

are certain investigators[7] who fail to recognize any essential morphological differences between *Spirochæta refringens* and *Spirochæta balanitidis,* while others[8] hold that there are at least four distinct varieties which were indiscriminately described under the name of *Spirochæta refringens.* From this it is easily seen how difficult it is to establish the etiological relation of any one of those organisms to a given definite pathological condition with which it is associated.

The second difficulty is that up to the present we have been unable to isolate these organisms in pure culture and have, therefore, been unable to determine their pathogenicity. The same lack of clearness surrounds the organism of Róna and those of the others. It appears that only a systematic cultural study of all these organisms can clear up the situation and it is for this reason that I describe in this article a spiral organism that has been isolated and grown in pure culture from a rather slowly progressing phagedenic lesion on the external genitalia of a woman.

Material and Cultivation.—The entire left labium showed an enormous swelling with induration, redness, and slight edema. The surface was moist with a seropurulent discharge from the vagina. In the center of the indurated region was an ulcer two by four centimeters and this was covered along its thickened edge with whitish serofibrinous matter. When touched, the ulcer was painful and it bled readily. The lesion had been present for about ten days and was increasing slowly in area. Under local anesthesia a portion of the ulcerated tissue was removed and used for the cultivation experiments.

The tissue thus obtained was rinsed thoroughly in sodium citrate saline solution and then ground in a sterile mortar by adding a fresh lot of citrate solution. In the resulting emulsion, the dark-field microscope revealed numerous irregularly wavy organisms which had a motility of their own that was slow but unmistakable. The length of the spirochætæ varied between four and thirty

[7] Rille, *München. med. Wchnschr.,* 1905, lii, 1377; Kraus, A., *Arch. f. Dermat. u. Syph.,* 1906, lxxx, 255.

[8] Eitner, E., *München. med. Wchnschr.,* 1907, liv, 770; Richards, G. M. O., and Hunt, L., *Lancet,* 1906, i, 667.

microns, but was usually about fifteen microns; the width was about 0.75 of a micron. The number of waves varied greatly, some having as many as eight and others only one or two, and some were almost straight. The wave length also varied greatly in different spirochætæ. Very frequently the organism took the form of the interrogation point. The ends were definitely pointed although not sharply drawn out. The short spirochætæ bent their bodies very slowly almost to a semicircle and then stretched out suddenly with a whipping motion. Some forms moved the terminal portion of the body to the left and right very much as an earth worm moves its head. The organisms were stained by Giemsa's stain, but did not retain the Gram stain.

The cultivation experiments with the material described above were carried out by means of a special medium and procedure described by me[9] for obtaining pure cultures of *Treponema pallidum, Treponema mucosum,* and *Spirochæta refringens* directly from contaminated human materials. Briefly stated, a number of ascitic agar tissue tubes were inoculated with the emulsion by means of a sterile capillary pipette. After inoculation the tubes were covered with sterile paraffin oil and incubated at 37° C. After a few days the stab canals of these tubes were filled up with the dense whitish colonies of the bacteria (chiefly staphylococci), but the rest of the agar column remained perfectly clear. At the end of one week some tubes commenced to show a very faint haze at various points along the stab canal, suggesting a colony of the pallidum. The intensity and size of the haze gradually increased, until, after about two weeks, there was an unmistakable growth of some organism. I left the tubes undisturbed for four weeks and then examined them to determine the cause of this hazy appearance. When the hazy portion of the media was taken out with a capillary pipette and examined under the dark-field microscope it showed a mass of rather heavy spiral organisms and a large number of round refractive bodies. Some of the organisms still showed distinct, apparently normal outlines, while others were granular and partly disintegrated. The round bodies were doubtless derived from the breaking up of these partly disintegrated

[9] Noguchi, H., *Jour. Exper. Med.*, 1912, xv, 90, 466.

forms. Transplants were made at once to a series of fresh culture tubes (ascitic agar tissue medium) in order to purify the culture. After several successive transplants a pure culture of the organism was finally obtained, and during the ten months that have elapsed since its purification the culture has passed through twenty sub-cultures.

Properties of Pure Cultures.—In the ascitic agar tissue medium a faint haze appears around the tissue within forty-eight hours and gradually extends upwards. The density of the growth increases, and within ten days it can be recognized without difficulty (figure 1). The organisms grow at 37° C., but not at 15° C. The strain is still transplantable after having been cultivated for three months at 37° C.

The length of different organisms varies considerably. In young cultures the majority of the spirochætæ measure ten to fifteen microns and show only one or two waves (figures 2, 3, 4, and 5). In old cultures many organisms attain a length of twenty to thirty microns, and the waves are more distinct and numerous (figure 6). The curvature and alternation of the waves are very irregular, and the changes in form are very gradual (figures 7, 8, and 9). The width of the body measures about 0.7 to 0.8 of a micron and is not always uniform throughout the entire length. In a long form there may be seen a nodular swelling and constriction at the middle or at each third of the body. The organisms usually have ends that are fairly pointed, although they may end obtusely. Young forms are frequently straight, but they may bend their bodies into parabolic curves, and then suddenly straighten out again. They also have a sluggish forward movement like that of a creeping worm. The terminal portion of the body is quite flexible and is moved like a feeler. Longer individuals are less motile. There is no flagellum, terminal projection, or undulating membrane. In a very old culture the majority of the organisms become granular and finally disintegrate into small fragments. At the same time there appear a large number of spherical bodies measuring 1.5 microns in diameter. These round bodies are often attached to the ends or the sides of short forms that are still well preserved (figures 10 and 11). When stained with Giemsa's solution these bodies show a small dot

of chromatic material at one pole, but do not take the ordinary bacterial spore stains. Another interesting feature of the organisms is that, under certain undetermined cultural conditions, they produce during disintegration numerous round or ovoid bodies of varying sizes, some as large as three microns in diameter, scattered singly, in pairs, or in clusters (figure 12). At one side of the protoplasmic mass of these bodies a careful examination reveals the presence of a highly refractive spot. In some of the larger forms there are two of these spots. In certain cultures there were transitions between the spherical bodies and those that contain one or two highly refractive spots in their more solid appearing protoplasm. The highly refractive points of the latter take a deep red stain with Giemsa's solution. Forms were also observed which showed more or less irregular refraction in different parts of the spiral body. This was possibly due to cross-bar concentration of the chromatin before the granular segmentation of the organism. In a stained preparation these refractive portions take a deep nuclear stain.

The organism is difficult to stain with most anilin dyes and does not retain the Gram stain. With Giemsa's solution it stains red.

It is a strict anaerobe and requires for its growth the presence of fresh sterile tissue in the ascitic fluid agar (the serum of rabbit or sheep is unsuitable). The protein constituents of the tissue and ascitic fluid are not visibly altered through its growth, but a distinct putrefactive sour odor somewhat resembling butyric acid develops in the culture.

Pathogenicity.—When introduced intradermically into a *Macacus rhesus* monkey and into rabbits an acute inflammation follows within twenty-four hours, but the reaction subsides in about three days without causing an ulcerative process. In the testicles of rabbits it causes a temporary induration which disappears completely within forty-eight hours.

Identification of the Organism.—Since the recent investigations of Gonder,[10] Gross,[11] Schellack,[12] Novy,[13] von Prowazek,[14] Keys-

[10] Gonder, R., *Centralbl. f. Bakteriol., Orig.*, 1909, xlix, 190.
[11] Gross, J., *Mitt. a. d. zoöl. Station z. Napel*, 1910, xx, 41.
[12] Schellack, C., *Arb. a. d. k. Gsndhtsamte*, 1908, xxvii, 364; 1909, xxx, 351.
[13] Novy, F. G., and Knapp, R. E., *Jour. Infect. Dis.*, 1906, iii, 291.
[14] von Prowazek, S., *Centralbl. f. Bakteriol., Orig.*, 1908, xlvi, 229.

selitz,[15] Swellengrebel,[16] and others, the classification of a spiral organism has become considerably more difficult. These authorities have shown that typical spirochætæ may fail to possess all the characteristics formerly regarded as differentiating the spirochætæ from the spirilla; *e. g.*, longitudinal fission, presence of an undulating membrane, absence of flagellum, etc.

In differentiating spirochætæ from spirilla, the importance once attributed to the mode of division has been still further decreased by the discovery of Schmeidlechner,[17] that a group of bacilli multiplies by longitudinal division.

The flexibility of the body was also once considered to be characteristic of spirochætæ, but the organisms of the relapsing fevers (hitherto called spirochætæ or spirilla indiscriminately) seem to have as much flexibility as any of the spirochætæ, and yet on account of the fact that they are supposed to divide transversely, they have been called spirilla by certain authorities (Novy,[18] Fraenkel,[19] Borrel,[20] Levaditi,[21] etc.).

It seems that spirochætæ, and some varieties of spirilla, have certain features that are possessed by the protozoa, and others that are characteristic of the bacteria. They occupy, therefore, an intermediary position between the protozoa and bacteria.

All the various spirochætæ that I have studied have shown features which are more highly differentiated than those seen in bacteria. For example, in most of the spirochætæ we observe during certain periods of their life the secretion of a small round body that stains like chromatin. The organisms often concentrate the chromatin material at one part of the body and then undergo a peculiar segmentation. The granules thus liberated seem to remain alive and at certain periods develop into spiral forms. Again, at

[15] Keysselitz, G., *Arb. a. d. k. Gsndhtsamte,* 1906, xxiii, 566.

[16] Swellengrebel, N. H., *Compt. rend. Soc. de biol.,* 1907, lxii, 213; *Ann. de l'Inst. Pasteur,* 1907, xxi, 448, 562; *Centralbl. f. Bakteriol., Orig.,* 1909, xlix, 529.

[17] Schmeidlechner, K., *Ztschr. f. Geburtsh. u. Gynäk.,* 1905, lvi, 291.

[18] Novy, F. G., and Knapp, R. E., *loc. cit.*

[19] Fraenkel, C., *Berl. klin. Wchnschr.,* 1907, xliv, 681; *Centralbl. f. Bakteriol., Orig.,* 1908, xlvii, 471; *München. med. Wchnschr.,* 1907, liv, 201.

[20] Borrel, A., *Compt. rend. Soc. de biol.,* 1906, lviii, 138.

[21] Levaditi, C., *Ann. de l'Inst. Pasteur,* 1906, xx, 593, 924.

certain stages of their life some of the spirochætæ become banded or dotted with the chromatin substance.

These primitive nuclear phenomena and also the distinct differentiation of the cytoplasm of some species into ectoplasm and entoplasm appear to me sufficiently characteristic to hold these organisms together under the group of spirochætæ without classifying them either with the bacteria or with the protozoa. Upon the basis of the conception just outlined I place my organism among the spirochætæ, but should further investigation make it possible to distinguish between spirochætæ and spirilla, and should it be evident that the organism is wrongly classified I should like to be permitted to correct the error.

Returning now to the question of whether the organism here described has been previously found by other investigators, it appears that the spirochætæ reported by Róna and Polland bear a certain resemblance to my organism and may possibly be identical with it, but I am not justified in concluding that this is the case, and to do so might be misleading; for if my spirochæta is different, I should be giving wrong cultural and biological properties to their organism. In order to avoid such confusion I propose to call my organism *Spirochæta phagedenis,* a conventional name merely indicating its source, but not necessarily showing its etiological relation to the lesion. In the event that this particular organism is found constantly in the phagedenic ulcers on the external genitalia and not in other conditions, its etiological significance would be established. Through lack of material I have up to the present been unable to pursue this subject further.

CONCLUSIONS.

1. A hitherto undescribed spiral organism has been isolated in pure culture from a case of mild phagedenic ulcer on the external genitalia of a woman. For this organism the name *Spirochæta phagedenis* is proposed.

2. *Spirochæta phagedenis* is a strict anaerobe and grows in the presence of fresh tissue in ascitic agar. It produces no apparent change in the media, but a somewhat offensive odor develops in the culture tube.

3. *Spirochæta phagedenis* incites a slight inflammatory reaction in the skin of a *Macacus rhesus* monkey and in the skin and testicles of rabbits.

4. Its etiological relation to the phagedenic lesions on the external genitalia has not yet been determined.

EXPLANATION OF PLATES.

PLATE 32.

FIG. 1. A stab culture of *Spirochæta phagedenis* in ascitic agar tissue medium, three weeks old, at 37° C.

FIGS. 2 and 3. Smears from a pure culture, three weeks old, stained with Giemsa's solution.

PLATE 33.

The dark-field appearance of *Spirochæta phagedenis* from pure cultures.

FIGS. 4 and 5. Young forms with few waves.

FIG. 6. An average form with shallow, irregular curves.

FIGS. 7, 8, and 9. An entangled mass of organisms, with occasional spore-like spherical bodies.

FIGS. 10 and 11. Formation of spherical bodies which are seen still attached to the organisms or already in the free state.

FIG. 12. Plasma granules in an old culture.

FIG. 13. *Spirochæta refringens* from a pure culture (for comparison).

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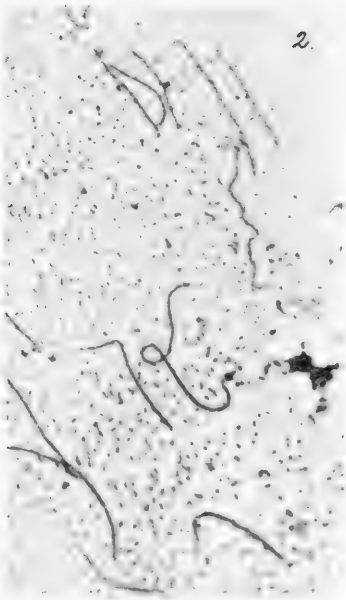
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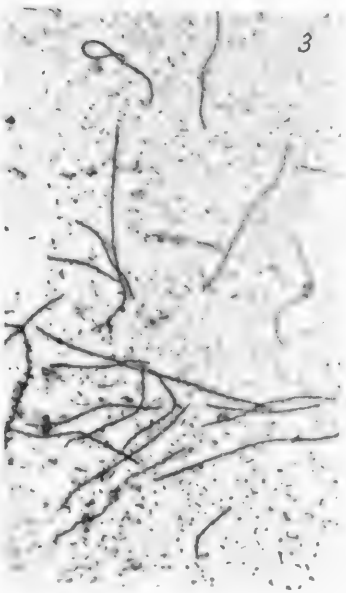
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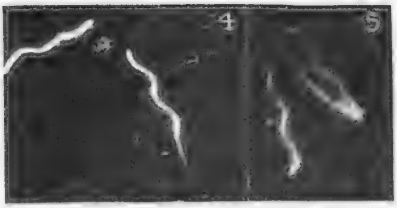
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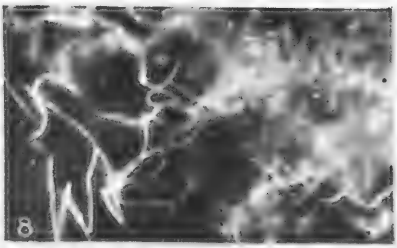
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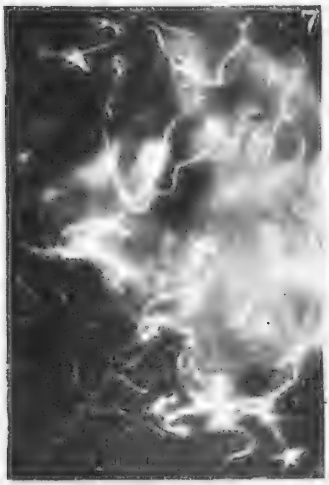
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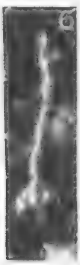
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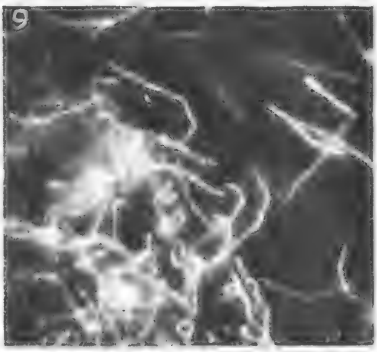
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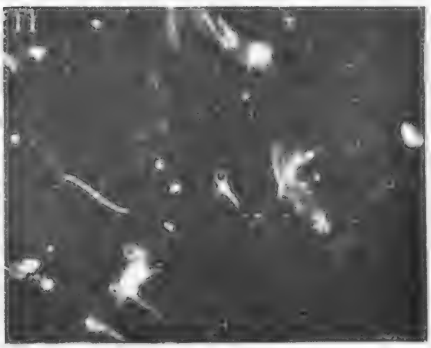
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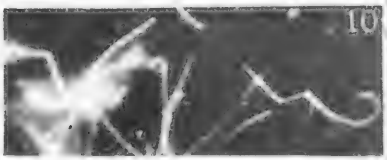
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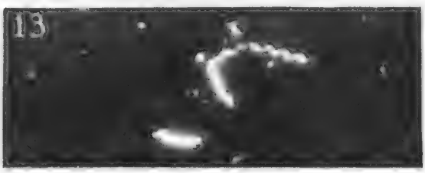
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STUDIES IN FERMENT ACTION.

II. The Extent of Leucocytic Proteolysis.*

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(*From the Morris Institute for Medical Research, Chicago.*)

In the course of studies which we are making on the relation between ferment action and certain immunity processes, questions arose as to the nature of the proteolytic activity of leucocytes. Much work has been done on the presence of proteolytic ferments of leucocytes, but few inquiries have been made as to how such ferments act or what relation they bear to other proteolytic ferments, such as trypsin and pepsin.

Friedrich Müller (1), in his study of the autolysis of the pneumonic lung, showed the presence of amino acids. Feissinger and Marie (2) found leucin and tyrosin in the end-products of the digestion of egg albumin by leucocytes. Jochmann and Lockemann (3) used a glycerin extract of the alcoholic precipitate of autolyzed leucocytes, and found that such an extract digested Witte's peptone to amino acids (tyrosin crystals, tryptophan). Erben (4) and Schumm (5) found peptone and albumose in the blood serum of patients with myelogenous leukemia due, they thought, to the splitting action of ferments derived from the leucocytes. In all of Opie's (6) work, which will be discussed more fully later, only once is mention made of the products of proteolysis; *i. e.*, the presence of peptone in the pleural fluid three days after the injection of turpentine.

Most workers (see Wiens (7)) have assumed that the demonstration of a leucocytic ferment capable of splitting protein was equivalent to demonstrating trypsin, and practically all studies on the so called antiferment of the blood serum have tended to show that trypsin and the leucocytic ferment are one and the same. Such findings are given by Jochmann and Kantorowicz (8). Injecting animals with either leucoprotease or with trypsin produces in the serum a substance capable of inhibiting the action of either ferment. This inhibiting substance is presumed to be a true antibody, and since it acts alike on trypsin or on leucoprotease, the two antigens are identical. Further than this, all the work done by Müller, Jochmann, Wiens, and Kantorowicz with the Müller plate method has shown simply the presence of a proteolytic ferment.[1]

* The first paper in this series, "A Chemobiological Study of the Relations of Pepsin to So-called Antipepsin," by Walter W. Hamburger, appeared in *The Journal of Experimental Medicine*, 1911, xiv, 535. Received for publication, May 17, 1912.

[1] A long review of this work and a complete bibliography is given by Wiens (7).

From this brief review of the literature it will be seen that we were confronted with the view that leucoprotease is a tryptase; we were, therefore, much surprised at finding in our first experiments some striking differences in reaction between trypsin and leucoprotease. In these experiments we attempted to compare the total incoagulable nitrogen of the digestion mixtures with the amount of amino acids as determined by the Sorenson method of formol titration. The figures for total incoagulable nitrogen were practically equal, while formol titration gave constantly a much higher figure for trypsin than for leucoprotease, indicating that trypsin carried digestion further than did leucoprotease.

In the preliminary work we used dried powdered leucocytes, and compared their action with that of powdered trypsin of commerce. If leucoprotease and trypsin are identical, the products of their digestion should be identical, and they should withstand the same treatment, so we studied first the comparative action of commercial trypsin and powdered leucocytes. From the very beginning it was evident that these ferments are not identical, and the results of all of our experiments were strikingly uniform.

Since our results depend in great part on the methods employed, it will be necessary to go into the technique in some detail.

Preparation of Trypsin.—Pure pancreatin (United States Pharmacopeia, Sharp and Dohme) was further purified by precipitation by acid alcohol from a weakly alkaline solution, extracting with physiological salt solution, and reprecipitating with alcohol. This purified powder was soluble in physiological salt solution and was so used in all experiments.

Leucocytic Ferment.—Inflammatory pus containing polymorphonuclear leucocytes and almost no other cellular elements was sent immediately after removal from the operating rooms of the hospital to the laboratory. The cells were then freed immediately by centrifugalization from any accompanying fluid, and were washed three times in physiological salt solution. The pus was then washed once with alcohol and dried with ether. A large amount of this dried powder was kept in stock in sealed dark bottles.

The first experiments deal entirely with the alkaline ferments which were extracted from the dried powder by sodium carbonate. N/20 sodium carbonate solution was added to a given quantity of the powder and left at 55° C. for one hour; then this was diluted with physiological salt solution to N/50 and allowed to stand for one hour, and finally was diluted to N/200 sodium carbonate. A little toluol was added and the mixture was left at 37° C. over night. When ready for use the next day the toluol was removed, the mixture was centrifugalized, and the faintly cloudy supernatant fluid was employed.

Dosage.—Preliminary experiments showed that in the digestion of beef serum as measured by incoagulable nitrogen, 0.004 gm. of purified trypsin was equivalent to 0.04 gm. of leucoprotease. Therefore, in using trypsin, 0.004 gm. was dissolved in 5 c.c. of physiological salt solution; and the leucoprotease was made up so that 5 c.c. of N/200 sodium carbonate contained 0.04 gm. of the original powder.

Chemical Methods.—In working out chemical methods our object was to measure large fractions of the digestion mixture, so that proportions could be compared. The fractions we attempted to estimate were: (1) total incoagulable nitrogen; (2) proteoses; (3) peptones; (4) amino acids. The test for trytophan was also done.

The technique we finally employed gave us four divisions, not exactly as outlined, but ones which for purposes of comparison were just as useful. After the necessary time (as given in the individual protocols) had elapsed, the coagulable protein, representing the undigested nitrogen, was precipitated by acidifying with a mixture containing:

Acetic acid	5 c.c.
Sodium chloride	10 gm.
Water	100 c.c.

The contents of the flask were then brought to the boiling point and filtered. The filtrate from this was neutralized in order to precipitate the acid albumin; but as we never found a precipitate on neutralization, this step was subsequently omitted, and the acid filtrate was used as follows.

Five or ten c.c. were withdrawn and used for the bromin water test for tryptophan. Many comparative titrations were made on the whole mixtures before precipitation, and on their corresponding filtrates, in order to see whether the precipitation changed the figures given by formol titration. As no change was produced by this procedure, and as formol titration is easier to perform on the filtrate, this method was adopted.

The remaining filtrate was then divided into two equal portions. On one half a Kjeldahl determination was made for total incoagulable nitrogen. The other half was saturated at the boiling point with crystals of sodium sulphate, filtered hot, and washed with a saturated aqueous solution of sodium sulphate. The precipitate represents the proteose fraction, and the filtrate represents all products of digestion below the proteoses. For the sake of simplicity this fraction will be called the peptone fraction. Kjeldahl determinations were made on the proteose fraction and on the peptone fraction, so that for each digestion mixture four fractions were measured.

In all the protocols the figures given represent the number of cubic centimeters of N/10 sulphuric acid neutralized by the distillate, and the result of the formol titration is correspondingly expressed in cubic centimeters of N/10 sodium hydrate. In order to make the results of some comparative value, the figures are given for 100 c.c. of original digestion mixture. Although this method does not give absolutely accurate results, it serves very well its purpose of giving comparative values, and is not difficult of execution.

One source of error was fairly constant. The figures found for the proteose

and peptone fractions should theoretically have equalled those for the total incoagulable nitrogen, but in many instances they exceeded them. A partial explanation for this error is found in the fact that the presence of the sodium sulphate accelerates the oxidation and gives, therefore, a somewhat larger figure for nitrogen. Furthermore, any small error was multiplied greatly by the method of expressing the results, so that this apparent discrepancy was, in fact, not very great.

General Procedure.—Each experiment was planned as a whole with the necessary controls. The zymolite[2] was made up *in toto* for each experiment and divided into flasks to which the ferments were added. The flasks were then incubated, the temperature and the duration of incubation being indicated in the protocols. In all instances toluol was added to each flask to prevent bacterial growth. The experiments that we cite are merely examples that were repeated and verified.

Experiment 1.—The zymolite used in this experiment was casein, and this was made up as a 1 per cent. solution in 0.1 per cent. sodium carbonate solution. To flask 1, containing 100 c.c. of casein solution, were added 10 c.c. of physiological salt solution containing 0.008 gm. of trypsin; to the second flask, containing 100 c.c of casein solution, were added 10 c.c. of N/200 sodium carbonate in physiological salt solution containing the extract of 0.08 gm. leucoprotease. A third flask contained 100 c.c. of casein solution, and a fourth contained 10 c.c. of leucoprotease extract. Digestion continued for forty-eight hours at 37° C. The results of this experiment are given below; for simplicity in expression the control figures are not given, but corrections have been made in expressing the results.

	Flask 1. Trypsin.	Flask 2. Leucoprotease.
Incoagulable nitrogen	80.0	84.0
Protcose	14.0	40.0
Peptone	75.0	49.0
Amino acids	10.0	1.0
Tryptophan	+	0

From the results of this experiment it is seen that, when measured by the incoagulable nitrogen, leucoprotease carried the digestion as far as trypsin, or slightly further. This indicated that the amounts of ferments used corresponded. The amino acid fraction with leucoprotease was, however, practically nil. The proteose fraction with leucoprotease was considerably greater than with trypsin, and the peptone fraction was correspondingly smaller. Further, the tryptophan reaction was positive with trypsin and negative with leucoprotease. This means that digestion with leucoprotease as performed in this experiment did not progress as far as the tryptic digestion.

[2] Loevenhart's (9) suggestion that the word zymolite be used instead of substrate is followed in this paper.

The part played by the duration of the digestion was investigated in experiments 2 and 3.

Experiment 2.—This was like experiment 1 except that the digestion was interrupted at the end of six hours.

	Flask 1. Trypsin.	Flask 2. Leucoprotease.
Incoagulable nitrogen	36.5	37.5
Proteose	8.5	25.0
Peptone	35.0	18.0
Amino acids	5.0	2.0
Tryptophan	+	0

The results in experiment 2 were practically identical with those in experiment 1. The total digestion was considerably less, but the proportions were about the same.

Experiment 3.—This was like experiments 1 and 2, but the digestion was allowed to proceed for seven days.

	Flask 1. Trypsin.	Flask 2. Leucoprotease.
Incoagulable nitrogen	60.0	65.0
Proteose	2.0	18.0
Peptone	60.0	59.0
Amino acids	17.0	5.0

In experiment 3 the tryptophan test was not made. In this experiment the differences between the digestion by trypsin and that by leucoprotease were not so great as in experiments 1 and 2; but with the leucoprotease the low figure for amino acids persisted and the proteose fraction was higher.

Zymolites other than casein were next used and a typical result with beef serum is given in experiment 4.

Experiment 4.—The serum here used was made up as a 25 per cent. solution in physiological salt and heated to 75° C. for one half hour before being used. The other details of the experiment correspond with those of the experiments just outlined. The control flask without ferment always contained a certain amount of proteose and peptone, for which corrections were made. Digestion continued for forty-eight hours at 37° C.

	Flask 1. Trypsin.	Flask 2. Leucoprotease.
Incoagulable nitrogen	88.0	63.0
Proteose	18.0	39.0
Peptone	75.0	35.0
Amino acids	20.0	6.0
Tryptophan	+	0

With the exception that the total incoagulable nitrogen was comparatively lower with leucoprotease in experiment 4 than in experiment 1, the results in the two corresponded exactly; the proteose fraction was high, the peptone low, the amino acid practically zero, and the tryptophan reaction negative.

Precipitated egg albumin was used, but proved to be poorly adapted for our experiments. However, the results, while not so definite as with the two other zymolites, were, in a general way, similar.

Experiment 5.—Purified egg albumin made up as 0.4 per cent. solution in N/200 sodium carbonate. Digestion at 37° C. proceeded for six days.

	Flask 1. Trypsin.	Flask 2. Leucoprotease.
Incoagulable nitrogen	21.0	11.2
Proteose	4.0	8.0
Peptone	26.0	4.0
Amino acids	6.0	0
Tryptophan	0	0

The small amount of digestion that occurred here greatly magnified the apparent errors, but the main points were the same as in previous experiments. With leucoprotease the proteose fraction was comparatively high, the peptone fraction comparatively low, and the amino acid fraction was zero.

Using Witte's peptone as a zymolite, trypsin was found to produce a high figure for amino acids and a markedly positive tryptophan reaction, whereas leucoprotease had no digestive activity at all. The importance of this observation will be emphasized subsequently in the second part of the paper.

Summary.—All these experiments were conducted with an alkaline extract of leucocytes which had been washed previously in salt solution and dried after washing with alcohol and ether. Such an extract contains a proteolytic ferment which, however, differs from trypsin in not carrying proteolysis so far. The amount of proteoses formed by this ferment exceeds greatly that found in true tryptic digestion, whereas the products of digestion below the proteoses are formed to a lesser extent. These facts, coupled with the inability of the leucoprotease to act on peptone, show that the ferment procured

by alkaline extraction of dried leucocytes does not split to any extent beyond the proteose stage, and is, therefore, not identical with trypsin. If, however, the facts in the literature previously cited are true, untreated leucocytes contain a ferment capable of such splitting, and this must have been lost in the process of treating the pus.

Experiments were then started with fresh dog leucocytes obtained by injecting aleuronat into the pleural cavity of dogs and, after twenty-four hours, bleeding the animals to death under ether anesthesia. The exudate obtained in this manner contains some red blood cells, but consists mainly of polymorphonuclear leucocytes (as was pointed out by Opie). The exudate was freed from serum by centrifugalizing and washed three times in physiological salt solution.

Experiment 6.—In this experiment 1 per cent. casein in 0.1 per cent. sodium carbonate made up in physiological salt solution was used as the zymolite. Flask 1 contained 50 c.c. of the casein solution and 0.004 gm. of trypsin; flask 2, 50 c.c. of casein solution and 5 c.c. of a 10 per cent. suspension of washed dog leucocytes in physiological salt solution; flask 3 contained 50 c.c. of physiological salt solution and 5 c.c. of a 10 per cent. suspension of cells. Digestion continued for forty-eight hours at 37° C.

	Flask 1. Trypsin and casein.	Flask 2. Leucocytes and casein.	Flask 3. Leucocytes. (control).
Incoagulable nitrogen	71.0	85.0	14.0
Proteose	8.0	30.0	10.0
Peptone	62.0	55.0	7.0
Amino acids	11.0	11.0	3.0
Tryptophan	++	+	0

This experiment differs from those quoted above, for the digestion with leucocytes here gives (1) a positive tryptophan reaction and (2) an amount of the amino acids equal to that found in the trypsin flask. The proteose fraction, however, is again somewhat higher than in the trypsin control. This experiment indicates the presence in fresh leucocytes of a ferment capable of splitting proteoses and peptones, and further proof of this action is found in the digestion of Witte's peptone.

Experiment 7.—Witte's peptone in 1 per cent. solution was used as the zymolite. To 50 c.c. of the peptone solution were added 10 c.c. of a 10 per cent.

suspension of washed leucocytes. In order to compare results with those previously given, the contents of the flask were made alkaline (0.2 per cent. sodium carbonate). Another flask containing ingredients which were the same, but which had been boiled at once, served as control. Digestion continued for five days.

	Flask 1. Leucocytes (fresh).	Flask 2. Leucocytes boiled (control).
Incoagulable nitrogen	55.0	60.0
Proteose	2.0	26.0
Peptone	50.0	36.0
Amino acids	9.0	4.0
Tryptophan	+	0

A comparison of these two columns of figures shows that the flask containing the live leucocytes underwent digestion to the amino acid stage; that is, living leucocytes contain a ferment capable of splitting Witte's peptone. This was evidence of the presence in fresh leucocytes of an ereptic ferment which is lost somewhere in the process of drying. As is well known, and as is seen from the control figures, Witte's peptone contains a comparatively large amount of proteose and some amino acids.

These experiments show that proteolysis by leucocytes is not a simple affair, and the next step in the problem was to determine, if possible, the mechanism of the reaction. Opie's (10) analysis of leucocyte ferments revealed two: (1) leucoprotease derived from the polymorphonuclear leucocytes and acting in an alkaline medium, and (2) lymphoprotease, derived from cells originating in lymphatic tissue and acting in an acid medium. The latter ferment loses its activity on being heated to 70° C. for one half hour, and is also somewhat impaired in the process of drying. All of Opie's work was done on native proteins as zymolites, and the extent of digestion was measured by the incoagulable nitrogen.

In our next series of experiments we attempted to determine whether the ereptic ferment of our experiments is simply Opie's acid-acting lymphoprotease, or whether it is a different ferment. In these experiments both fresh dog leucocytes and washed pus were used and all experiments were performed at 55° C. under toluol.

Experiment 8.—In this experiment, the effect on the proteolytic activity of fresh dog leucocytes of an acid and an alkali, and also the effect of heat (70° C. for half an hour) were studied. Beef serum, 25 per cent. in physiological salt

solution, was used as the zymolite, and a 10 per cent. suspension of fresh washed dog leucocytes was employed.

Flask 1 contained serum 50 c.c., plus leucocytes 10 c.c. The reaction of the flask was equivalent to 0.2 per cent. sodium carbonate.

Flask 2 contained serum 50 c.c., plus leucocytes 10 c.c. The reaction of the flask was equivalent to 0.2 per cent. acetic acid.

Flask 3 was the same as flask 1, except that the leucocytes were heated to 70° C. for half an hour.

Flask 4 was the same as flask 2, except that the leucocytes were heated to 70° C. for half an hour.

Flask 5 contained serum 50 c.c., plus leucocytes 10 c.c., boiled immediately.

	Flask 1. (Alkali.)	Flask 2. (Acid.)	Flask 3. (Alkali and heat.)	Flask 4. (Acid and heat.)	Flask 5. (Heat alone.)
Incoagulable nitrogen	24.0	20.0	20.0	10.0	10.0
Proteose	15.0	Lost	9.0	6.0	6.0
Peptone	10.0	10.0	10.0	4.0	4.0
Amino acid	7.0	6.0	3.0	1.0	1.0
Tryptophan	+	+	0	0	0
End reaction	Alkaline	Acid	Alkaline	Acid	?

In this experiment several interesting and important points may be noted. The fresh leucocytes acted in acid medium only to a slight degree less than in an alkaline medium, and in each instance proteolysis extended to the amino acids. One might infer from this that only one ferment is present, and that this one is capable of acting in either alkaline or acid medium, but comparison of the figures for flasks 3 and 4 shows that this is not the case. In these two flasks the leucocytes had been heated to 70° C. for one half hour. In flask 3 there is still considerable digestion in an alkaline medium, while flask 4 shows the same results as the control. In other words, heating had completely inhibited the action of the acid-acting ferment, while it had only slightly impaired the action of the ferment acting in an alkaline reaction.

Almost as high a figure was obtained for incoagulable nitrogen in flask 3 as in flask 1, but in flask 3 the amino acids were diminished and the tryptophan test was negative. This means that the peptone-splitting action of the ferment active in alkaline solution was partly destroyed by heating to 70° C. That this action may be due to the acid-acting proteose must be considered, since both are destroyed at 70° C., but this assumption is definitely disproved by the results in

flask 1, where it is seen that, although ereptic digestion occurred, the end reaction was alkaline. Consequently this peptone-splitting ferment is active in an alkaline medium and must be different from the acid-acting ferment.

These deductions were tested further by using Witte's peptone as a zymolite. This experiment was performed at the same time and under the same conditions as experiment 8.

Experiment 9.—Flask 1 contained peptone 1 per cent., 50 c. c., plus leucocytes 10 c.c. The reaction of the flask was equivalent to 0.2 per cent. sodium carbonate.

Flask 2 contained peptone 1 per cent., 50 c.c., plus leucocytes 10 c.c. The reaction of the flask was equivalent to 0.2 per cent. acetic acid.

Flask 3 was the same as flask 1; the leucocytes were heated to 70° C. for half an hour.

Flask 4 was the same as flask 2; the leucocytes were heated to 70° C. for half an hour.

Flask 5 contained peptone and leucocytes that had been boiled immediately.

	Flask 1. (Alkali.)	Flask 2. (Acid.)	Flask 3. (Alkali and heat.)	Flask 4. (Acid and heat.)	Flask 5. (Heat alone.)
Incoagulable nitrogen	89.0	91.0	95.0	72.0	72.0
Proteose	39.0	28.0	34.0	28.0	34.0
Peptone	53.0	68.0	63.0	48.0	45.0
Amino acids	12.0	16.0	8.0	7.0	5.0
Tryptophan	+	+	0	0	0
End reaction	Alkaline	Acid	Alkaline	Acid	?

Although the figures given here may seem at first sight somewhat confusing, it must be remembered that Witte's peptone is a mixture of proteoses, peptones, etc., in unknown proportions. The amino acid figures are, therefore, the most important indices of digestion, and it can be seen that we may make the same deductions here as were made from experiment 8. An ereptic ferment is present in fresh leucocytes, and this is not the same as the acid-acting lympho-protease of Opie. The ereptic ferment is active in either an alkaline or an acid medium.

Subsequent experiments were undertaken with the dried powdered leucocytes to determine whether under conditions other than those imposed in the first experiments ereptic activity might be demonstrated. These experiments showed that whether the leucocytes were used in alkali, acid, or neutral medium, ereptic activity was not

demonstrable. They showed further that the acid-acting protease was still present, thus giving additional evidence for the view that ereptase and the acid-acting protease are not identical.

SUMMARY.

This work proves that the proteolytic action of leucocytes is not a simple cleavage by one ferment. There are present two proteases, one acting in an alkaline medium and the other in an acid medium. This confirms Opie's work. In the leucocytes there was found in addition an ereptic ferment which is capable of acting in an acid or alkaline medium. Since this action is lost by heating to 70° C., and by drying and keeping the leucocytes, it probably represents a ferment which acts on the digestion products of the two proteases. Analogy with other ferments in the body confirms this opinion.

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A COMPARISON OF SIMULTANEOUS POLYGRAPH AND MICROGRAPH TRACINGS.*

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INTRODUCTION.

Since the original description of the micrograph,[1] its practical value in clinical medicine has been studied in this department. In a large series of records from normal and pathological cases we have been able to make tracings with the micrograph that give all the information that one can obtain with the polygraph, and we are convinced that the micrograph gives even more information.

In former papers Crehore called attention to two groups of small waves, occurring in the apex, carotid, and jugular tracings, which have a definite relation to the valvular events in the cardiac cycle; e. g., the A- and B-groups, and also an M-wave,[2] and in a study of the presphygmic period, he described a presphygmic wave, P, in apex curves, corresponding to the M-wave in the jugular, and preceding the closure of the auriculo-ventricular valves.

From these observations we were led to study polygraphic and micrographic records, and to attempt to answer the following questions: 1. Does the micrograph register the movements of the heart and vessels more accurately than the polygraph? 2. Does the micrograph do away with the errors introduced by the inertia of the slowly responsive levers and diaphragm of the polygraph? 3. Are the valvular events shown in the micrographic records? 4. Are delicate movements in the heart and vessels registered by the micrograph, which are not shown by the polygraph; for example, thrills and murmurs?

* Received for publication, May 18, 1912.

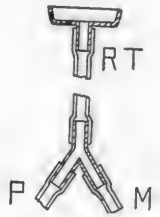
[1] Crehore, A. C., and Meara, F. S., *Jour. Am. Med. Assn.*, 1911, lvi, 1549; *Jour. Exper. Med.*, 1911, xiii, 616.

[2] Crehore, A. C., *Jour. Exper. Med.*, 1911, xiv, 351, 520.

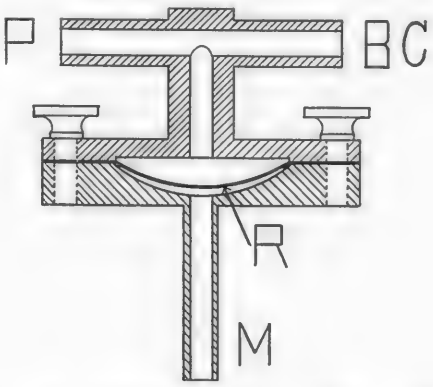
It occurred to us that for comparison simultaneous records with the two instruments could be made.

DESCRIPTION OF METHOD.

Simultaneous records were obtained by connecting the rubber tube leading from the receiving jugular tambour to the main stem of a Y-tube (text-figure 1). Of the two rubber tubes connected with the two branches of the Y-tube, one was joined to the polygraph recording tambour, and the other to the micrograph.



TEXT-FIG. 1. The method of taking simultaneous jugular or apex tracings with the polygraph and the micrograph. *RT* is the receiving tambour connected to the Y-tube; *P* is connected to the polygraph recorder, and *M* to the micrograph recorder.



TEXT-FIG. 2. Crehore tambour for brachial tracings. When using this for simultaneous Erlanger and micrograph brachial records, connect a rubber tube from the Erlanger instrument to *P*, and from the brachial cuff to *BC*. The air in the space above the diaphragm, *R*, which is of soft flexible rubber, is thus at the same pressure as the air in the cuff, while the pressure on the opposite side of the diaphragm to which the micrograph is connected at *M* is at the atmospheric pressure. The soft rubber deflected by the pressure forms the segment of a sphere.

To obtain simultaneous brachial records from the two instruments, the tube from the brachial cuff of the Erlanger-Hirshfelder polygraph was connected to a Crehore tambour (text-figure 2), one tube leading from this to the Erlanger manometer and the other to the micrograph.

The brachial records were taken at a pressure of 10 to 20 mm. below systolic pressure.

The micrograph curves were plotted in the usual way,[3] and for comparison the polygraph records were enlarged to the same time-scale as the micrograph records. This was done by making upon photographic plates contact prints (negatives) from the smoked paper record of the polygraph. These negatives were then placed in a projection lantern and the tracings were thus thrown upon a screen. By varying the distance of the screen from the lantern the degree of magnification could be made as great as was desired. In this manner the magnification was made approximately equal to the time-scale of the micrograph record, and was then traced by hand directly upon the screen. This method, as far as we know, has never been used before for enlarging polygraphic tracings.

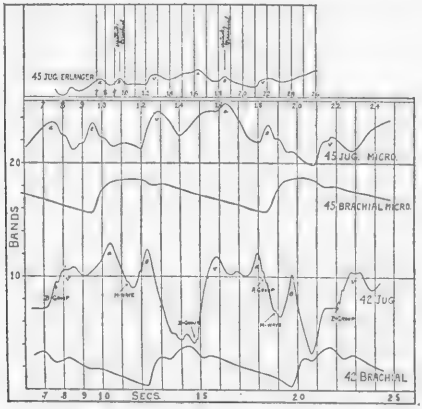
ANALYSIS OF THE RECORDS.

By the method described the time-scales were made alike, so that each record traveled the same distance in one second of time. It was then of interest to compare the amplitudes of the curves obtained with each instrument. It is evident from the method of magnification that the amplitude of the polygraph tracing was magnified in proportion to the enlargement of its time-scale, but even then the excursion obtained with the polygraph was not so great as that obtained with the ordinary diaphragm of the micrograph, the same receiving tambour being used for both instruments. In the polygraph there is no means of increasing the amplitude of the excursion if this is desired, but by using a thinner diaphragm in the micrograph the amplitude of the excursion may be multiplied many times.

The test of the instruments is, however, not the amplitude of the excursion which each can produce, since by sufficient magnification they may always be made equal, but rather the ability to record without distortion the pressure changes which are transmitted to them by the receiving tambour. It is from this point of view that we should compare the following records made simultaneously by the two instruments.

[3] Crehore, A. C., and Meara, F. S., *Jour. Exper. Med.*, 1911, xiii, 616.

Records 45 and 42 (*Text-figure 3*).—The four records of No. 45 were taken simultaneously. The two lower curves were made by the micrograph, and the upper curves by the polygraph, the brachial of the polygraph being shown only by lines marking the time of the beginning of the upstroke. The speed of the paper when the polygraph record was taken was about 0.28 of an inch per second, while the speed of the film in the micrograph was 8.68 inches per second. That is, the micrograph film moved about 31.2 times faster than the polygraph paper. The Erlanger jugular record in this instance was magnified about 19.5 times by the lantern. This magnification was not quite sufficient to make the time-scales



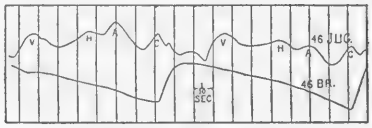
TEXT-FIG. 3. Records 45 and 42.

of the two records equal. The reason for the insufficient magnification in this instance was that there was not enough distance available in the room between the lantern and the screen.

The vertical lines mark one tenth second intervals, and corresponding points in the jugular records are seen to occur at the same instant. A v-wave, for instance, is just beginning at the 1.2 second mark. The jugular record of the micrograph shows the typical normal phlebogram, in which the A-, C-, and V-waves appear much more distinct than in the one made with the polygraph. Even though the magnification were sufficient to make the time scales equal, the amplitude of the micrograph record would still be the greater of the two.

When simultaneous records were taken, using a Y-tube as in text-figure 1, approximately one half of the available energy was used by each instrument, each losing the energy diverted to the other. Record 42 was taken with the micrograph alone on the patient used for record 45, the polygraph being disconnected. This had the effect of greatly increasing the amplitude of the excursions, and brings out additional details in the jugular tracing. In this record the typical B-group just preceding the v-wave in the jugular is seen. The M-wave in the fall of the jugular A is distinctly shown and the A-group in the fall of

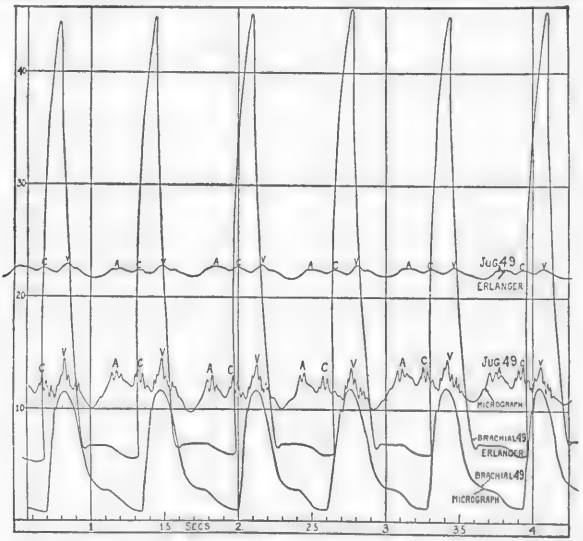
the jugular A is also shown. The jugular record as a whole is very distinct, and is better than the ordinary polygraph record.



TEXT-FIG. 4. Record 46.

Record 46 (*Text-figure 4*).—In this record simultaneous polygraph tracings were also taken, but are not shown. Note the well marked A-, C-, V-, and H-waves in the jugular.

Record 49 (*Text-figure 5*).—In this record are shown four tracings all taken simultaneously, two with the polygraph, and two with the micrograph. These records enable us to compare accurately the performances of the two instru-



TEXT-FIG. 5. Record 49.

ments. In the tracings of the polygraph the effects of the inertia of the lever and of friction[4] upon the smoked paper are plainly seen. The speed of the film

[4] The effect of friction is much greater than one would suppose. As illustrating this, the example of the siphon recorder may be cited. This is the instrument now in commercial use for receiving long submarine cable messages The writing is done by a small glass tube called the siphon which carries the

in the micrograph was about 6.5 inches per second, and in this figure the time-scale of the polygraph is magnified about 23.5 times, which makes it equivalent to the scale of the micrograph.

In this case the jugular records, which were taken from the same receiving tambour, may be compared directly. The amplitude of the micrograph record as a whole is much greater, but the important point is that the inertia of the lever and the friction upon the paper have almost completely obliterated the more rapid waves which were superposed upon the normal A-, C-, and V-waves.

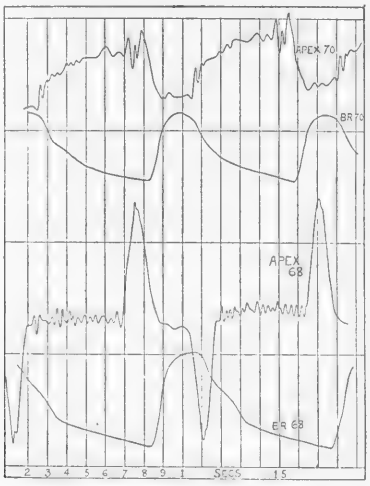
In this patient there was a well marked thrill over the jugular bulb, a fact which was verified by all observers. The micrograph record taken from this point shows strikingly what we have observed in other patients having definite thrills over the heart or vessels, but this is not shown in the record from the polygraph. The frequency of these waves was roughly thirty-five per second, a value much too low to be attributed to the period of the diaphragm of the micrograph.

While we hesitate to offer an explanation of these waves it seems quite probable that they are produced in the vessels by vibrations, which give rise clinically to a palpable thrill, and which are detected by the stethoscope as booming, low pitched sounds. Their frequency corresponds to a very low bass note.

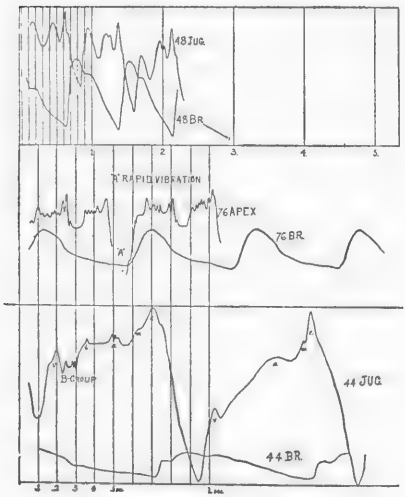
The extraordinary effect of inertia is again shown in the polygraph brachial record. This was taken from a case of aortic insufficiency, and the typical effect of the water-hammer pulse is seen in the polygraph record. Note the immense fling of the lever, its sudden fall, and the distortion of the dicrotic notch. This was exaggerated by the difference in the tambours used (text-figure 2). In the micrograph record of the brachial, there is seen the rapid rise of the wave, a definite notch before the summit is reached, and

ink in a continuous stream to the record slip of paper. In this instance the available energy is so little that if the siphon were allowed to drag on the paper the wavy line which constitutes the record would be obliterated due to the friction alone. It was not until means were devised to spurt the ink from the point of this siphon upon the paper without the siphon touching the paper that this became a practical instrument for receiving messages. Usually in this instrument sufficient vibration is given to the siphon tube to cause it to discharge the ink.

a well marked dicrotic notch; these are not shown in the polygraph record.



TEXT-FIG. 6. Record 68.

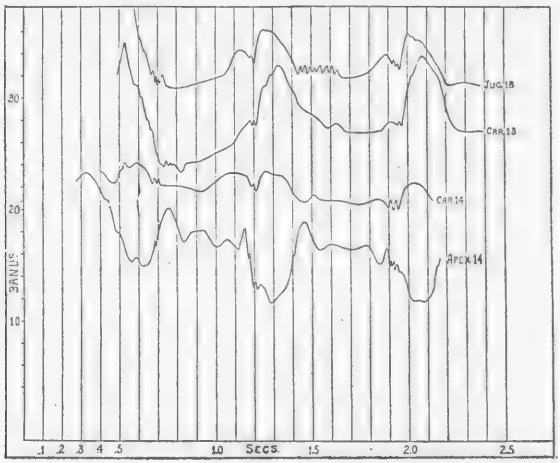


TEXT-FIG. 7. Record 76.

For comparison we give some records from cases of mitral stenosis, with definite thrills over the precordium.

Record 68 (*Text-figure 6*).—The small waves preceding the apex rise are extraordinary in this tracing, which shows graphically what was noted with the stethoscope as a rather low pitched murmur and by the palpating hand as a strong presystolic thrill, occupying the whole diastolic period. These vibrations lasted 0.55 of a second. The speed of the film was 8.68 inches per second.

Record 76 (*Text-figure 7*).—A case of mitral stenosis and insufficiency. The small waves are especially well shown, both during systole and diastole.



TEXT-FIG. 8. Record 18.

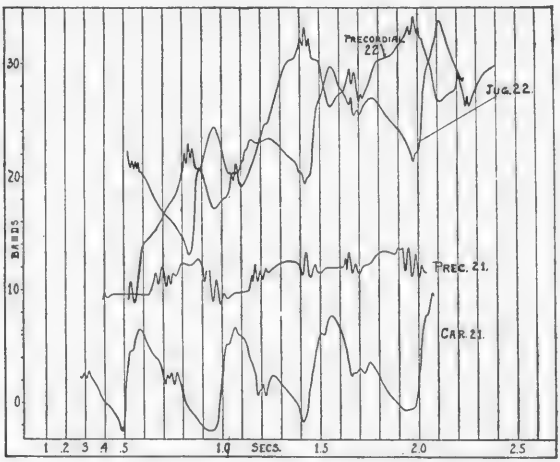
Record 18 (*Text-figure 8*).—This was taken from a typical case of mitral stenosis showing an early diastolic and presystolic murmur.

Record 21 (*Text-figure 9*).—This was from a patient with aortic and mitral insufficiency. The curves were taken from the jugular and conus arteriosus (third left interspace, 1½ inches from the mid-sternal line), showing vibration of the valvular events in the A- and B-groups.

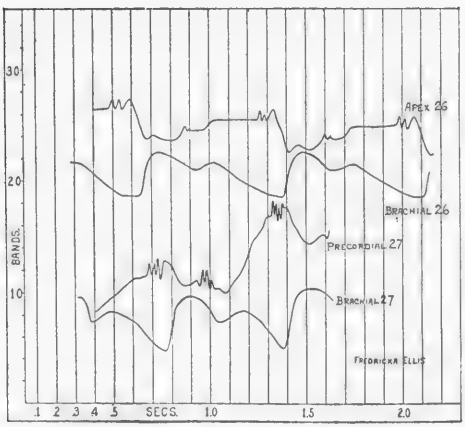
Records 26 and 27 (*Text-figure 10*).—These are from the patient used for record 21. The records were taken from the apex and brachial, and from the conus and brachial.

Records 54, 55, and 58 (*Text-figure 11*).—These were from a patient with rheumatic arthritis and acute myocarditis. There was marked arhythmia.

Record 54 gives four tracings taken simultaneously from the brachial and jugular, two with the polygraph and two with the micrograph. Note the extrasystole at 1 shown in both the micrograph and polygraph records. The corresponding jugular shows this to be the ventricular type of extrasystole, with the A-wave absent. A careful study of the longer polygraph record shows this form of extrasystole to be fairly frequent. This record shows the normal A-, C,- and V-waves in the jugular except at the points where extrasystole has occurred.



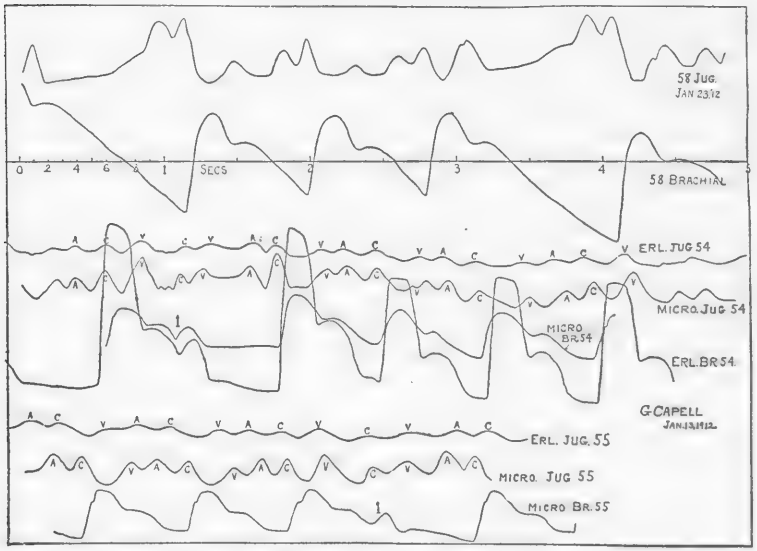
TEXT-FIG. 9. Record 21.



TEXT-FIG. 10. Records 26 and 27.

Record 55 shows one extrasystole. The polygraph record is given for comparison. In records 54 and 55 note the sluggish rise of the polygraph jugular as compared with the same rise in the micrograph records. In the latter the waves are well defined and are much larger.

Record 58 was taken from the patient used for records 54 and 55, but was made ten days later. Here the normal A-, C-, and V-waves are seen in the jugular. The polygraph record is not shown, since it corresponds closely with the micrograph record. The arhythmia here originated in the sinus.



TEXT-FIG. 11. Records 54, 55, and 58.

SUMMARY.

From a study of the accompanying tracings we may conclude that:

1. The delicate mechanism of the micrograph registers the movements in the heart and blood-vessels more accurately than does the polygraph.

2. The inertia of the polygraph lever and diaphragm distorts the tracing. The difference between the results obtained with the two instruments is shown by the records made simultaneously with the micrograph and the polygraph.

3. The micrograph is particularly useful for recording heart sounds. Some records illustrating this are shown. We are direct-

ing especial attention to this and will report the results in a subsequent paper.

4. While the micrograph will not supersede the polygraph or electrocardiograph, we feel that it has distinct possibilities as a clinical instrument.

A STUDY WITH THE ELECTROCARDIOGRAPH OF THE MODE OF DEATH OF THE HUMAN HEART.*

By G. CANBY ROBINSON, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research, New York.)

PLATES 34–38.

The changes in the mechanism of the heart-beat which occur at the time of death have been repeatedly observed experimentally in animals, and from these observations it has been generally believed that that part of the heart in which the property of rhythmicity is the most highly developed is the part that ordinarily continues to beat longest as the heart dies. The evidence seems conclusive that it is in the region where the superior vena cava joins the right auricle that rhythmicity is most highly developed, and that it is in this region that the stimulus of the heart-beat originates. Here it is, in other words, that the cardiac pace-making region is situated, and this function belongs apparently to the specialized tissue found along the sulcus terminalis, which constitutes the node of Keith and Flack.

Koch[1] has questioned the belief that this region in the right auricle, the ultimum moriens, is the last part of the heart to die in man, and he bases his objections on observations on four human fetal hearts which returned to activity after the chest was opened soon after birth. He believed that the region of the coronary sinus was the last part of the human heart to die. Hering[2] criticizes his findings and says that the incisions made in the hearts were responsible for the lack of agreement between Koch's observations and his experiments.

Rohmer[3] made electrocardiographic studies of fatal cases of diphtheria and reported that in three complete dissociation of auricles and ventricles occurred. He found an unusual form of ventricular complex which he thought was due to damage to the myocardium. His paper with curves has not as yet been published. Koch and Rohmer have furnished the only observations which deal directly with the changes in the mechanism of the dying human heart.

* Received for publication, May 23, 1912.

[1] Koch, W., *Beitr. z. path. Anat. u. z. allg. Path.*, 1907, xlii, 203.

[2] Hering, H. E., *München. med. Wchnschr.*, 1909, lvi, 845.

[3] Rohmer, *München. med. Wchnschr.*, 1911, lviii, 2358.

The present study is based upon electrocardiographic records obtained from seven patients before and during the actual stoppage of the heart. There were two cases of poliomyelitis, one of pneumococcus meningitis, and four cases of lobar pneumonia. Death in the cases of poliomyelitis occurred from paralysis of the respiratory muscles, the children being otherwise in relatively good physical condition. As wires run throughout the hospital, records could be taken without moving or disturbing the patients in any way. The second lead was used in each case, the German silver electrodes being attached to the right arm and left leg by bandages wet with salt solution.

I sought to obtain records of the cardiac mechanism just before the occurrence of death and to continue taking records at frequent intervals as long as any cardiac activity persisted. There were naturally many failures to obtain records, especially when fatalities occurred suddenly; while success in some instances followed only after hours of careful watching with the electrodes in place and the galvanometer in operation.

Clinical death was considered to have occurred when respiration finally ceased, when no heart sounds could be heard, and when muscular relaxation and the general appearance of the patient indicated to the physician that death had occurred. Not until all these conditions were fulfilled was the patient considered dead. The electrocardiograms have been analyzed in order to determine the changes in the mechanism of the heart-beat in relation to the time of clinical death in each case. The rate of the heart-beat has been calculated; the conduction time from auricles to ventricles (P–R time), and in some cases through the ventricles as indicated by the duration of the QRS complex, and the length of ventricular systoles (R to end of T) have been measured in seconds. The heights of the various waves have been measured in millimeters and the results of these analyses are seen in the tables.

Case 1.—A child, eighteen months old, suffered with poliomyelitis and died of paralysis of the respiratory muscles. The heart sounds could be heard occurring regularly for three minutes after respirations ceased, and clinical death is considered to have occurred when the heart sounds were no longer audible. The rate of the heart-beat gradually declined from 132, eleven minutes before death (figure 1), to 45, ten minutes later. The cessation of respiration produced no

TABLE I.

Case 1. Age 18 months. Poliomyelitis. Curve No. 134.

Number of curve.	Number of figure.	Time.	Rate per minute.		Conduction time in seconds.		Length of ventricular systole in seconds.	Height of waves in mm.		
			Auricular.	Ventricular.	P-Q.	Q to end of R.	Q to end of T.	P.	R.	T.
134.3	1	11 min. a.m.[4]	132.0	132.0	0.10	0.057	0.24–0.26	1.4	11.5	2.0
		8 min. a.m.	113.0	113.0	0.11	0.053	0.25	1.3	11.2	2.0–2.5
		5 min. a.m.	59.0	59.0	0.09–0.10	0.066	0.30–0.31	1.3	10.5	3.2
		4 min. a.m.	54.7	54.7	0.12	0.066	0.31	1.3	10.0	3.5–4.0
		3 min. a.m.	54.0	54.0	0.12	0.066–0.077	0.30–0.315	1.0	10.0	3.0
		Respiration ceased here without affecting electrocardiogram.								
		1 min. a.m.	45.0	45.0	0.14	0.068	0.34	1.0	9.0	2.5
134.11	2	$8\frac{1}{2}$ min. p.m.	—	22.6	——	——	0.26	—	11.0	5.0
		Auricles not beating.								
134.12	3	10 min. p.m.	39.0	17.3	——	0.083	0.29	1.0	9.5–10.0	5.5
		Complete block.								
		11 min. p.m.	0	15.8	——	0.093	0.30	—	9.0	5.5–6.0
		Auricles not beating.								
134.14	4	$11\frac{1}{2}$ min. p.m.	28.5	15.4	——	——	0.29	—	8.0–8.5	5.5
		To show change in complex.								
		$11\frac{3}{4}$ min. p.m.	Occasional beat.	16.2	——	——	0.27–0.28	—	8.0	5.0–5.5
		$12\frac{1}{2}$ min. p.m.	Occasional beat.	18.2	——	——	0.311	—	6.5	—
		$13\frac{1}{2}$ min. p.m.	Occasional beat.	22.2	——	——	0.29	—	6.5	—
		14 min. p.m.	Occasional beat.	26.0	——	——	0.29	—	7.5	—
		$14\frac{1}{2}$ min. p.m.	Occasional beat.	—	——	——	0.28	—	8.0	—
		Long pause.								
134.22	5	$16\frac{1}{2}$ min. p.m.	Occasional beat.	35.0	——	——	0.28	—	8.0	—
		$17\frac{1}{2}$ min. p.m.	—	37.0	——	——	0.30	—	8.0	—
		No movement of string after 18 minutes p. m.								

[4] In the tables a. m. stands for ante mortem, p. m. for post mortem.

appreciable effect. The conduction (P-R) time gradually lengthened from 0.10 to 0.14 of a second during this time. Records could not be obtained until eight and a half minutes after death on account of an accident to the instrument. Then the P-wave was absent, indicating cessation of auricular activity, the ventricles beating 22.6 times per minute (figure 2). Ten minutes post mortem the auricles were again beating regularly at a rate of 39 per minute, while the R- and T-waves indicated that the ventricles beat 17.3 times a minute, complete dissociation being present (figure 3). The auricles again ceased beating for a short time and then began again, at first at a rate of 28.5 (figure 4). Afterwards they beat only occasionally and irregularly until sixteen and a half minutes post mortem, when they finally ceased. The ventricles continued to beat until eighteen minutes post mortem and gradually increased in rate from 15.4 per minute, at eleven and a half minutes post mortem (figure 3), to 35, at sixteen and a half minutes post mortem (figure 5). This increase in rate seems to depend, however, at least in part, on the rest afforded the heart by the long pauses that occurred. In this case then the ventricles beat after the auricles had ceased. The curves reproduced from those obtained in this case show the characteristic changes in the ventricular complex which occurred in every case. These changes consist in a decrease in the size of the R-wave, an increase in the size of the T-wave, and a gradual fusing of these two waves. There is no striking change in the duration of ventricular systole in this case. The time of conduction of the impulse throughout the ventricles, as measured from the beginning of Q to the end of R, gradually lengthened from 0.057 to 0.093 of a second. On account of the fusion of R and T, it could not be measured later than eleven minutes post mortem (table I).

Case 2.—A child of four years, with poliomyelitis, died of respiratory paralysis. The time when the heart sounds ceased to be heard was not recorded, but on account of definite changes that took place suddenly in the electrocardiograms corresponding to changes that occurred in other cases at the time of clinical death, the time of these changes is inferred to be the time of clinical death.

For several hours before death the patient breathed with great difficulty, one half of the diaphragm alone supporting respiration. Four hours and a half before death, marked changes in rate occurred in a fairly rhythmic manner (figure 6). Diminution in the size of the P- and T-waves occurred with the periods of slow rate, producing an electrical complex resembling that seen when the vagus tone is raised (Rothberger and Winterberg[5]). It seems probable that these changes in rate and in the form of the complex are dependent on changes in vagus tone accompanying respiratory movements.

At the time when clinical death is inferred to have occurred, there was marked slowing of the cardiac rate and the auricular activity apparently ceased, the ventricles contracting alone at a rate of 61 per minute. The auricles were seen (figure 7) to become active once more, and when they set the pace of the ventricles again, the rate increased to 90 per minute. The auricles stopped beat-

[5] Rothberger, J., and Winterberg, H., *Arch. f. d. ges. Physiol.*, 1910, cxxxv, 506.

TABLE II.

Case 2. Age 4 years. Poliomyelitis. Curves 118–122.

Number of curve.	Number of figure.	Time.	Rate per minute.		Conduction time in seconds.		Length of ventricular systole in millimeters.[6] R to end of T.	Height of waves in mm.		
			Auricular.	Ventricular.	P–R.	R–S.		P.	R.	T.
119.7	6	60 hrs. a.m.	125	125	0.14	0.067	0.29	2.3	9.5	3.0
		10 hrs. a.m.	91	91	0.13	0.065	0.30	1.3	12.5	3.0
		4 hrs. and 35 min. a.m.	100	100	0.14	0.061–0.008	0.28–0.29	1.0–1.5	9.0	2.0–3.5
		There is a marked change of rate at this time. Changes in conduction through the bundle and through the ventricles do not go parallel.								
121.4	7	Death probably here.	——	61.8	——	——	11.0 mm.	——	5.0	3.0
			90.0	90.0			13.0 mm.	——	6.0	2.5
		Rate more rapid when auricles set the pace.								
		½ min. p.m.	Not active.	58.5	——	——	13.0 mm.	——	5.5	3.0
		¾ min. p.m.	57.6	57.6	——	——	11.0–12.0 mm.	——	——	——
		Not delayed. The auricles return and take up the pace in this curve after having ceased beating for 15 seconds. The rate is not altered.								
		5 min. p.m.	56.8	56.8	Delay in P–R time.		10.0 mm.	1.5	4.5	3.5
		12 min. p.m.	49.4	About 33 (irregular).	——	——	10.0 mm.	——	——	——
		There is partial block, 3 to 2 rhythm being present. Conduction much delayed when a beat gets through.								
		17 min. p.m.	——	21.4	——	——	10.6 mm.	——	7.5	2.0
		No evidence of auricular activity; ventricles regular.								
		18 min. p.m.	54.8	18.0	——	——	10.0 mm.	——	7.5	3.0
		19 min. p.m.	39.2	14.7	——	——	9.2 mm.	——	5.0–6.0	3.0–4.0
		Complete block typical.								
		19¼ min. p.m.	40.0	13.6 (irregular)	——	——	9.7 mm.	——	5.0	3.4
122.2	8	20 min. p.m.	40.0	Ventricular fibrillation.	——	——	——	——	——	——
		20¼ min. p.m.	40.0	One last ventricular wave.	——	——	6.5 mm.	——	5.0	——
		20½ min. p.m.	38.4	Not active.	——	——	——	——	——	——
		Auricles still beating presumably 20 minutes after clinical death.								

[6] These measurements were made in millimeters, instead of seconds, as the time-marker was not operating.

ing again for about fifteen seconds, and then the ventricular rate again sank to 58.5 per minute. The auricles began again three quarters of a minute after the time clinical death is inferred to have occurred and took up the pace at about the same rate as that at which the ventricles were already beating. The auricles then continued to beat almost constantly, decreasing slightly in rate, and were still active at a rate of 34 beats a minute in the last curve taken, twenty minutes after the inferred time of clinical death. The ventricles followed the pace of the auricles until twelve minutes after inferred clinical death, when, following a period of delayed conduction, partial block (3 to 2 rhythm) occurred. Complete heart-block occurred six minutes later, the ventricular rate being 18 per minute. This rate gradually decreased and at nineteen minutes after inferred clinical death, the ventricles beat irregularly 13.6 times a minute. One minute later ventricular fibrillation set in (figure 8), after which one ill formed ventricular complex occurred. There was a gradual but not marked shortening of the ventricular systole, as was indicated by the length of the ventricular complex in the electrocardiograms, and the characteristic change in its form occurred (table II).

Case 3.—A child of nine months, who died of pneumococcus meningitis. No tracings were taken until four minutes after clinical death, at which time the heart was beating at a rate of 40 per minute. The electrical complex was at this time abnormal, there being a diphasic P-wave representing the auricular activity, while the T-wave was much exaggerated and almost fused with the R-wave. The conduction (P-R) time gradually lengthened in the record made at this time, from 0.22 to 0.28 of a second. At five minutes post mortem the rate was 28. The P-wave had become negative and very small, while the conduction time had shortened, varying from 0.145 to 0.16. This seems to indicate that the point of origin of stimulus formation had moved to a point in the auricles nearer the atrioventricular junction. The R- and T-waves approached each other. At the end of the record a long pause of over six seconds occurred. At six minutes post mortem two beats occurred at the same rate as before. Both auricular and ventricular complexes were seen. In the latter the R- and T-waves were partly fused. No evidence of cardiac activity was seen in records made during the next few minutes, so in this case cardiac activity ceased six minutes after clinical death, the auricles and ventricles stopping synchronously (table III).

TABLE III.

Case 3. Age 9 months. Pneumococcus Meningitis. Curve 138.

Time.	Rate per minute.	Conduction time in seconds. P-R.	Length of systole in seconds. R-T.	Height of waves in mm.		
				P.	R.	T.
4 min. p.m.	40	0.22–0.28	0.24–0.28	1.3	6.0	5.0
	Diphasic P-wave.					
5 min. p.m.	28	0.145–0.20	0.20–0.27	—	6.0–7.5	3.5–4.0
	P-wave is negative and nearer the ventricular complex than before.					
6 min. p.m.	27	0.16	0.25	—	4.5	—
	Only two beats.					

Case 4.—A man of thirty-seven, who died of lobar pneumonia. The heart was beating at a rate of 136 per minute sixteen and a half hours before death and the electrocardiogram shows that the rhythm was regular except for an occasional auricular extrasystole. The first post-mortem record was not made until four minutes after clinical death, when the heart was beating irregularly and the electrocardiogram showed a very unusual form of arhythmia (figure 9). In the second group a positive P-wave was followed by a small R-wave, the P-R time being 0.15 of a second. Then a negative P-wave occurred, followed by a small R-wave, the P-R time being 0.24 of a second. Then after a long diastole, a large R-wave, which was not preceded by any evidence of auricular activity, occurred. This group was repeated several times. One large R-wave alternated with two small R-waves, the small R-waves alone being preceded by P-waves. Later these were always negative. From six to fourteen minutes post mortem the cardiac rate varied from 43 to 64 a minute in an irregular manner. At first there was no evidence of auricular activity, but at eight minutes post mortem there was a small negative P-wave preceding the R-wave by 0.123 of a second. The P-wave gradually increased in prominence, but remained negative, while the conduction (P-R) time gradually lengthened until it was 0.31 of a second (figure 10). After fourteen minutes post mortem there was no evidence of auricular activity, while the ventricles continued to beat for thirty-five minutes after clinical death. Their rate became gradually slower and

TABLE IV.

Case 4. Age 37 years. Pneumonia. Curve 179.

Number of curve.	Number of figure.	Time.	Rate per minute.	Conduction time in seconds.		Length of ventricular systole in seconds. R to end of T.	Height of waves in mm.		
				P-R.	R-S.		P.	R.	T.
		16 hrs. and 37 min. a.m.	136.4	0.13–0.14	0.075	0.315	2.2	9.5	2.0
179.2	9	4 min. p.m.	78.5	0.15–0.24	0.065–0.08	——	——	9.0–12.5	——
		A very unusual type of arhythmia seen.							
		6 min. p.m.	64.0	Auricles not active.	0.077	0.285	——	7.5	4.0
		8 min. p.m.	43.0	0.123	——	0.250	——	5.5	4.0
		Auricles return but P-wave is very small.							
		9 min. p.m.	46.0	0.24	——	0.230	Negative P-wave.	6.5	3.5
		10 min. p.m.	60.0	0.26	——	0.260	——	6.0	3.5
179.7	10	14 min. p.m.	54.0	0.31	——	0.240	——	6.0	3.0
		Last of auricular activity. Very long conduction time.							
		15 min. p.m.	40.6	——	——	0.220	——	6.5	3.0
		21 min. p.m.	36.0	——	——	0.240	——	7.5	2.5
		23 min. p.m.	30.0	——	——	0.240	——	8.0	—
		28½ min. p.m.	26.0	——	——	0.240	——	7.0	—
		Ventricles beating irregularly.							
		31½ min. p.m.	——	——	——	0.240	——	8.0	—
		33 min. p.m.	13.8	——	——	0.240	——	7.5	—
		33½ min. p.m.	6.3 seconds between the two beats.						
179.23	11	35 min. p.m.	1 beat only.		——	0.350	——	6.0	—

their activity was irregular after twenty-eight and a half minutes post mortem. Only a single but well defined ventricular complex was seen thirty-five minutes post mortem (figure 11). The characteristic fusion of the R- and T-waves took place gradually, while except for the final ventricular complex, which was abnormally prolonged, no marked change took place in the length of the ventricular systoles. In this case was seen the longest post-mortem cardiac activity that was observed (table IV).

Case 5.—A woman of twenty-five years, who died of pneumonia. No records were made until twelve minutes after clinical death, when the heart was beating 40 times a minute. The electrical complex was at this time abnormal, consisting of a fairly well defined P-wave, a deep Q-wave, and a small R-wave already fused with the T-wave. At thirteen minutes post mortem the rate was 35.6 and the conduction (P-Q) time was delayed (0.225 of a second, figure 12). The rate slowed gradually and the conduction time became slightly more delayed until at sixteen minutes post mortem the rate was 24.4 per minute, while the conduction time was 0.24 of a second. At seventeen minutes a long pause occurred, 13.6 seconds of which were recorded. This pause was followed by a very unusual ventricular complex which followed a notched P-wave after a conduction time of 0.28 of a second. The complex consists of a typical left ventricular, or apical complex followed by a right ventricular, or basal complex (figure 13). That these forms of complexes result from left and right ventricular contractions has been demonstrated by Nicolai[7] and others. This unusual form of complex suggests that first the left and then the right ventricle contracted separately. It was followed by a pause, eight seconds of which were recorded. The cardiac activity was again resumed and the heart continued

TABLE V.

Case 5. Age 25 years. Pneumonia. Curve 149.

Number of curve.	Number of figure.	Time.	Rate per minute.	Conduction time in seconds. P–Q.	Length of ventricular systole in seconds.	Height of waves[8] in mm. T.
		12 min. p.m.	40.0	—	—	—
149.2	12	13 min. p.m.	35.6	0.225	0.31	5.5
		14 min. p.m.	34.0	0.25	0.31	6.0
		15 min. p.m.	30.4	0.24	0.31	6.0
		16 min. p.m.	24.4	0.24	0.33	6.0
149.6	13	17 min. p.m.	Unusual type of complex suggesting hemisystole.			
					0.87	6.5
		18 min. p.m.	30.8	0.24	0.34	5.2
		19 min. p.m.	30.0	0.24	0.33	5.7
		20 min. p.m.	31.0	0.26	0.36	5.0
		21 min. p.m.	24.0	0.28	0.34	5.5
		22 min. p.m.	32.7	0.35	0.36	5.0
			A pause of 22 seconds preceded the foregoing curve.			
		23 min. p.m.	20.0	0.375	0.35	6.0
149.15	14	$25\frac{1}{2}$ min. p.m.	After a stoppage of $2\frac{1}{2}$ minutes.			
			33.0	0.42–0.54	0.40	0.35

[7] Nicolai, G. F., *Med. Klin.*, 1912, viii, 322.

[8] P and R not measurable.

to beat slowly but with marked changes of rate and long periods of complete cardiac standstill. The heart finally ceased beating twenty-five and a half minutes after clinical death. Just before the final record was obtained, a pause for two and a half minutes occurred. After this pause both auricles and ventricles again became active at a rate of 33 beats a minute. The conduction time in this record (figure 14) gradually lengthened until the ventricles failed, the auricles continuing for a short time at the same rate. The conduction time, which was already distinctly delayed at nineteen minutes post mortem, further lengthened and at twenty-three minutes post mortem it was 0.375 of a second. In the final record it increased beat by beat from 0.42 to 0.54 of a second. The form of the ventricular complex changed very little in this case, except that the duration of ventricular systole gradually lengthened (table V).

Case 6.—A woman of thirty-seven years, with pneumonia. Before death, electrocardiograms showed a heart rate of 156, a conduction time of 0.12, and ventricular systole of 0.25 of a second duration (figure 15). The first record obtained one minute after clinical death showed that the heart was beating regularly at a rate of 72 per minute with practically no change in conduction time (figure 16). At two minutes post mortem there was complete dissociation of auricles and ventricles, and groups of ventricular contractions seperated by long

TABLE VI.

Case 6. Age 37 years. Pneumonia. Curve 195.

Number of curve.	Number of figure.	Time.	Rate per minute.		Conduction time in seconds.		Length of ventricular systole in seconds. R to end of T.	Height of waves in mm.		
			Auricular.	Ventricular.	P–R.	R–S.		P.	R.	T.
195.2	15	52 min. a.m.	156.0	156.0	0.120	0.09	0.25	1.5–2.5	11.5–12.3	2–3
	14	min. a.m.	155.6	155.6	——	——	——	—	——	——
195.5	16	1 min. p.m.	72.0	72.0	0.128	0.08	0.265	1.5	11.0	3.0
		1½ min. p.m.	65.4	65.4	0.115	0.08	0.30	1.0	11.0	3.5
195.7	17	2 min. p.m.	Irregular. Occasional auricular activity. Last seen in this curve. Ventricles beating irregularly. Long pauses seen.							
195.8	18	2½ min. p.m.	——	168.0	——	0.08–0.094	0.30	1.5	9.5–10.3	2.5
			Ventricular tachycardia.							
		3½ min. p.m.	——	138.0	——	——	——	—	8.0– 8.5	2–3
			in small group. Ventricles beating in groups and isolated beats without auricles. Long pauses of several seconds occurred.							
		4 min. p.m.	——	47.4	——	0.10	——	—	7.0	2.0
			Ventricular rhythm established. Slight arhythmia.							
195.12	19	4½ min. p.m.	——							
			Ventricular fibrillation.							
				31.5						
		5 min. p.m.	Ventricles at first irregular and then rhythmic. Marked change in complex.						0.33–0.22	5.5
195.14	20	5½ min. p.m.		31.9					0.23	6.0
		6 min. p.m.	No beats.							
		6½ min. p.m.		27.0					0.23	6.0
		7 min. p.m.	One beat.							
				24.0						
		7½ min. p.m.	Two beats only.						0.20	5.3

pauses were seen. Auricular activity ceased altogether at this time (figure 17). Two and a half minutes post mortem a group of ventricular complexes occurred at a rate of 168 per minute, showing apparently true ventricular tachycardia (figure 18), while four minutes post mortem the ventricular rhythm established itself at a rate of 47.4 with but slight arhythmia. Four and a half minutes post mortem ventricular fibrillation of short duration set in (figure 19), and was followed for about one minute by a period of distinct arhythmia, but at a fairly slow rate. Five and a half minutes post mortem the ventricular rhythm again became regularly established at a rate of 32 per minute (figure 20). The ventricular complexes were markedly altered and showed the characteristic form seen in nearly all the cases observed just before cessation of ventricular activity. The rate then became slower and long periods without cardiac activity occurred. Two last beats, occurring at a rate of 24 per minute, were seen seven and a half minutes after clinical death. This case showed at first a lengthening and then finally a distinct shortening of the length of ventricular systole, which reached 0.33 of a second at five minutes post mortem and then shortened to 0.20 of a second in the last record (table VI).

Case 7.—A woman of thirty-four years, who died also of pneumonia. The electrocardiograms from this case indicated that the auricles had ceased to beat one minute before clinical death, while the ventricles continued for seventeen minutes after the patient ceased to breathe. The ventricles showed marked irregularities in rate, but two minutes post mortem they were beating regularly at 45.8 per minute. They beat at a rate of 107 times a minute thirteen minutes post mortem, showing a bigeminal type of arhythmia. They beat regularly at a rate of 47, sixteen minutes post mortem, but no evidence of cardiac activity was seen seventeen minutes post mortem. Because the records were not satisfactory, a more detailed analysis is not possible (table VII).

TABLE VII.

Case 7. Age 34 years. Pneumonia. Curve 228.

Time.	Rate.		
1 hr., 6 min. a.m.	120.0	Auricles active.	Ventricles regular.
1 min. a.m.	57.1	Auricles not active.	Ventricles regular.
½ min. a.m.	51.7		Ventricles regular.
2 min. p.m.	45.8		Ventricles regular.
4 min. p.m.	60.0		Ventricles nearly regular.
The type of ventricular complex changed here.			
11 min. p.m.	60.0	Ventricles beating irregularly.	
13 min. p.m.	107.0	Ventricles beating bigeminally.	
14 min. p.m.	71.0	Ventricles beating irregularly.	
15 min. p.m.	53.6	Ventricles beating regularly.	
16 min. p.m.	47.0	Ventricles beating regularly.	
No movement of string after 17 minutes post mortem.			

SUMMARY.

In four of the seven cases the ventricles remained active from one and a half to eighteen minutes after the electrocardiograms

failed to show evidence of auricular activity. In two cases the auricles outlasted the ventricles and in one case only did the auricles and ventricles stop apparently at the same time. Complete dissociation occurred three times. Some delay in the conduction time was seen in five of the seven cases. In two cases the auricles ceased to beat before evidence of impaired conduction appeared. There was always marked slowing; the slowest independent ventricular rates varied from 13.6 to 47.0. The slowest rates at which the auricles beat regularly varied from 20 to 65 per minute. There was never evidence of auricular fibrillation, although in two cases the electrocardiograms give fairly conclusive evidence that ventricular fibrillation occurred. The ventricles reëstablished a regular rhythm after a short period of ventricular fibrillation in one case, while in the other but one ventricular contraction occurred after the appearance of fibrillation.

Characteristic changes in the ventricular complex of the electrocardiograms occurred in all the records. They consisted of a gradual fusion of the R- and T-waves, forming, when the fusion was complete, a large rounded or peaked wave. In some cases the identity of the two waves was not entirely lost. In spite of the marked change in shape of the ventricular complexes, there was often but little change in their duration. In some cases the ventricular systole was shortened at the end, while in others it was prolonged. The change in the form of the ventricular electrical complex indicates that the course of the stimulus and the manner of the contraction of the muscle were abnormal. The fact that the R-wave became gradually prolonged suggests that the conduction of the stimulus through the ventricular walls became delayed as the heart died. The fact that after death there is a continuation of cardiac muscular activity sufficient to cause a difference in electrical potential between the two sides of the body does not necessarily mean that a ventricular systole in the sense of muscular shortening takes place. It has been observed experimentally that well defined electrical complexes may be caused by cardiac activity which cannot be seen or recorded graphically. As the duration of the ventricular complexes characteristic of the dying heart usually does not differ markedly from the duration of the complexes before clinical death, it seems probable

that the entire musculature of the ventricles participates in the contraction; as definite shortening, or at least a marked change in duration, would be expected if only a part of the ventricular musculature participated in the activity which produced the complex.

CONCLUSIONS.

In acute infectious diseases cardiac activity sufficient to give a definite record with the electrocardiograph may continue in the human heart for some minutes after clinical death has occurred. In the seven cases described the cardiac activity continued from six to thirty-five minutes after all the usual clinical signs of death had occurred. In four cases the ventricular outlasted the auricular activity; in two cases this was reversed; and in one case the two parts of the heart seemed to cease synchronously. Marked slowing of the rate of cardiac activity always occurred and there was usually distinct delay in the conduction time between auricles and ventricles. Complete dissociation was seen in three cases. Ventricular fibrillation occurred in two cases, in one of which the ventricles again established a regular rhythm. Evidence of auricular fibrillation was never seen. Characteristic changes in the ventricular electrical complex occurred in all cases. They consisted of a decrease in the size of the R-wave and an increase in the size of the T-wave, and a tendency to a fusion of these waves. There was usually but little change in the duration of the ventricular complexes as the cardiac activity gradually ceased. The foregoing observations indicate that when death occurs from an acute infectious disease there is no one point in the human heart which may be considered as the "ultimum moriens."

EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. Curve 134.3. Case 1.
FIG. 2. Curve 134.11. Case 1.
FIG. 3. Curve 134.12. Case 1.
FIG. 4. Curve 134.14. Case 1.

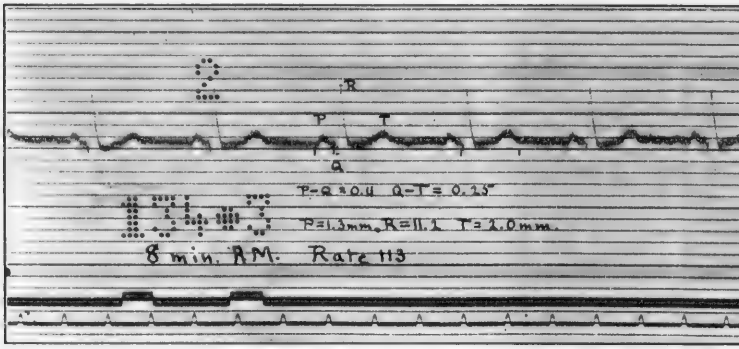
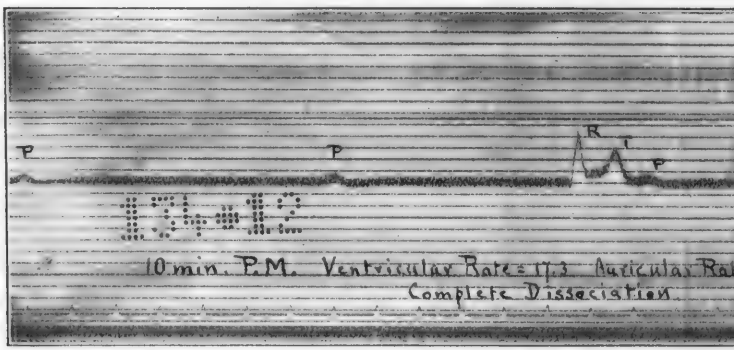
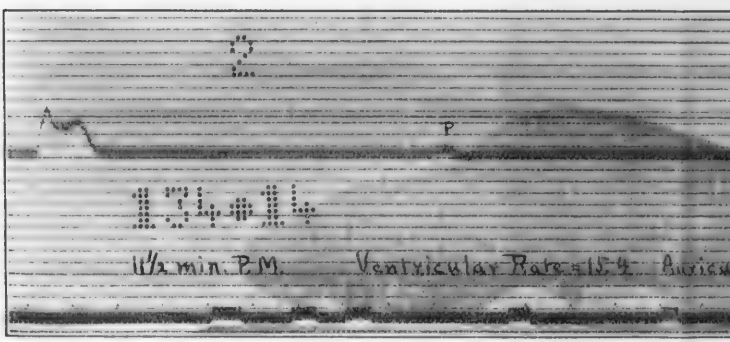
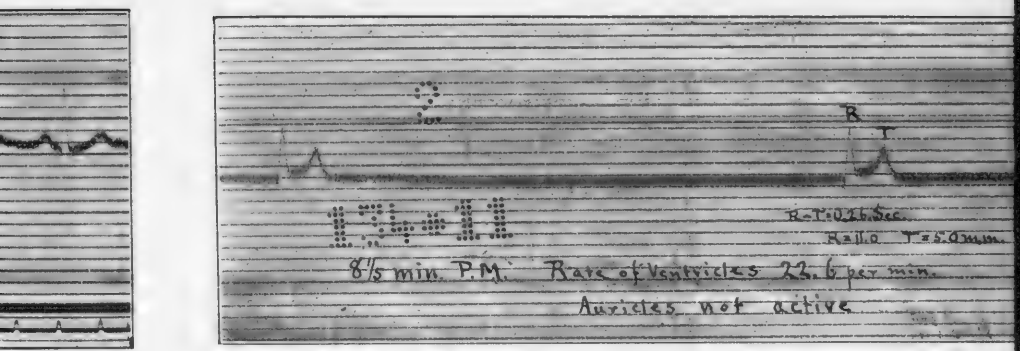

R
P
T
Q
P-Q = 0.14 Q-T = 0.23"
P=1.3mm. R=11.1 T=2.0mm.
8 min. P.M. Rate 113

FIG. 1.


P
P
R
T
P
10 min. P.M. Ventricular Rate = 17.3 Auricular Ra
Complete Dissociation


P
11½ min. P.M. Ventricular Rate = 15.4


R
T
R-T=0.26 Sec.
R=11.0 T=5.0mm.
8⅘ min. P.M. Rate of Ventricles 22.6 per min.
Auricles not active

P
P
R
T
P
R-T=0.29 sec.
=31.0 R=9.5 T=5.5mm.
=37 per min.

FIG. 3.

R T
P
P
R-T= 0.29
R=5.0 T=5.5mm.
Rate=28.5

FIG. 4.

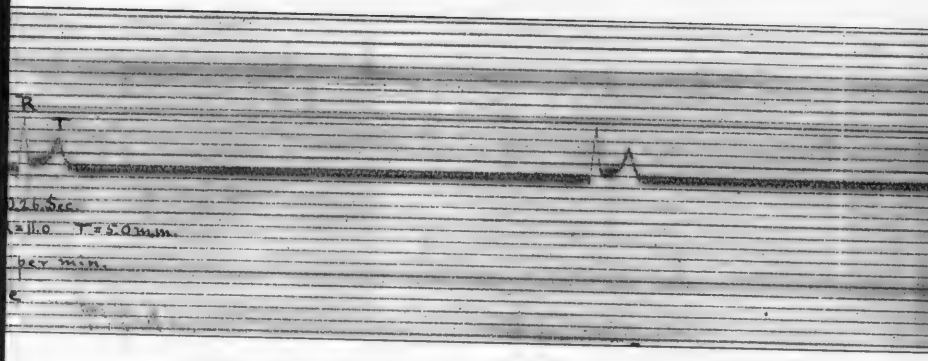
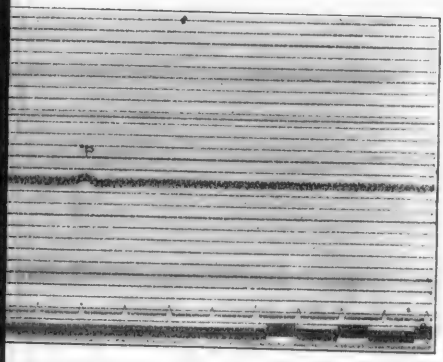


FIG. 2.



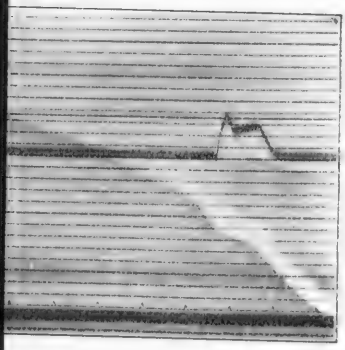
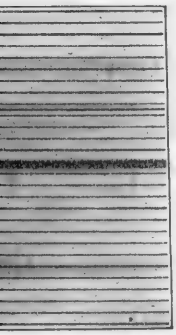


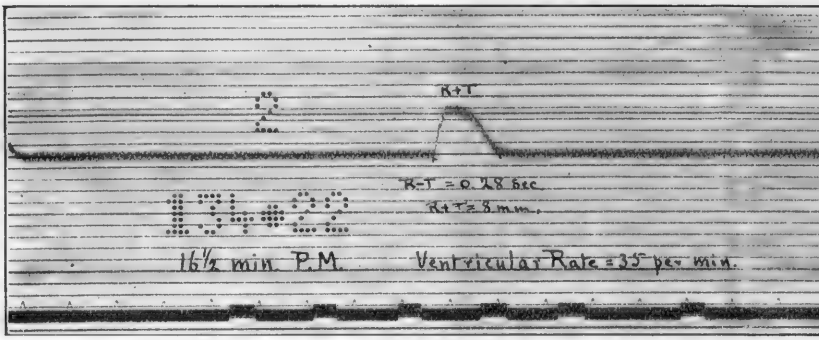
PLATE 34.

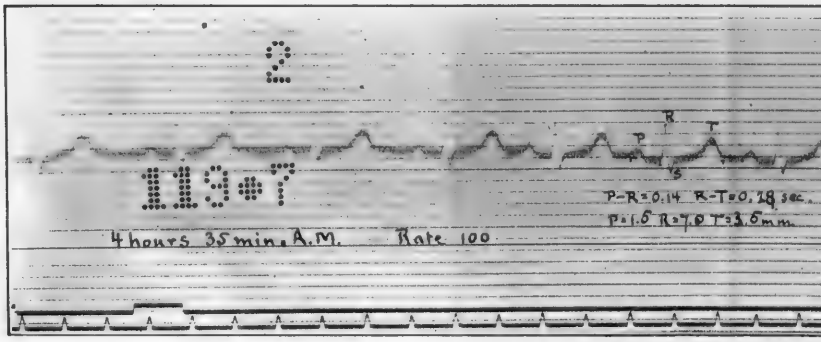


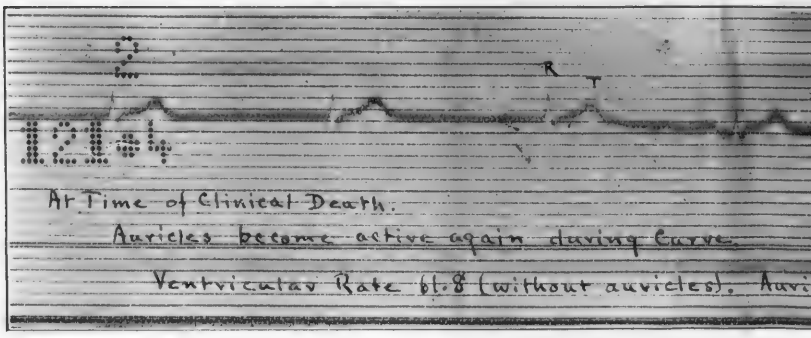
G. Canby Robinson.

PLATE 35.

FIG. 5. Curve 134.22. Case 1.
FIG. 6. Curve 119.7. Case 2.
FIG. 7. Curve 121.4. Case 2


R+T
R-T = 0.28 sec.
R+T = 3 mm.
16½ min. P.M.
Ventricular Rate = 35 per min.


P
R
T
P-R = 0.14 R-T = 0.28 sec.
P = 1.5 R = 7.0 T = 3.5 mm.
4 hours 35 min. A.M.
Rate 100


R
T
At Time of Clinical Death.
Auricles become active again during Curve.
Ventricular Rate 61.8 (without auricles). Auri

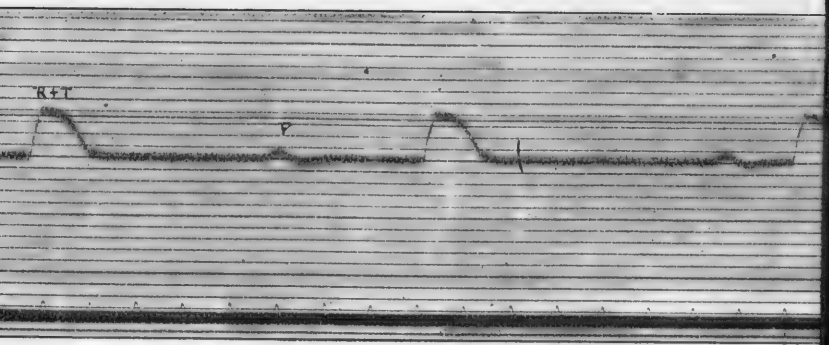


FIG. 5.

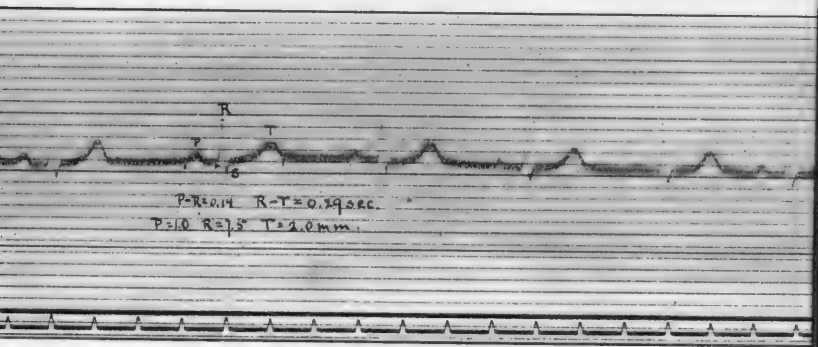


FIG. 6.

FIG. 7.

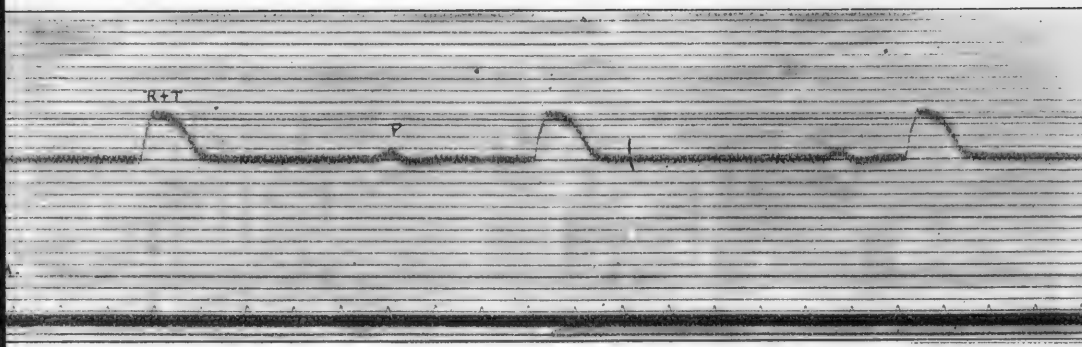


FIG. 5.

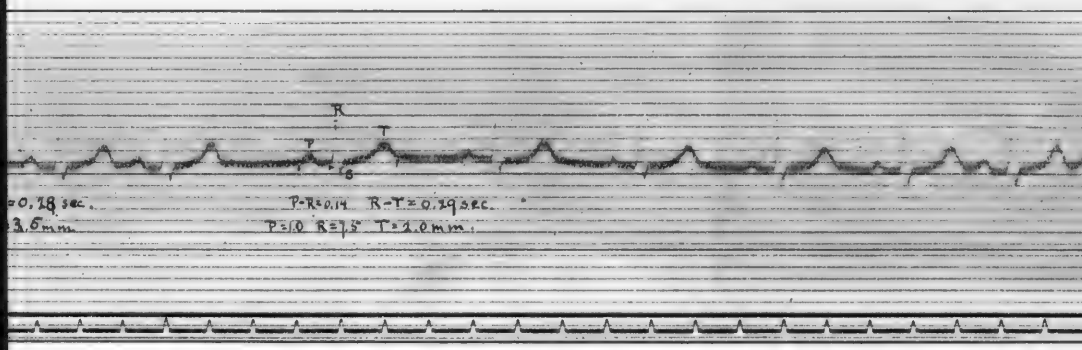


FIG. 6.

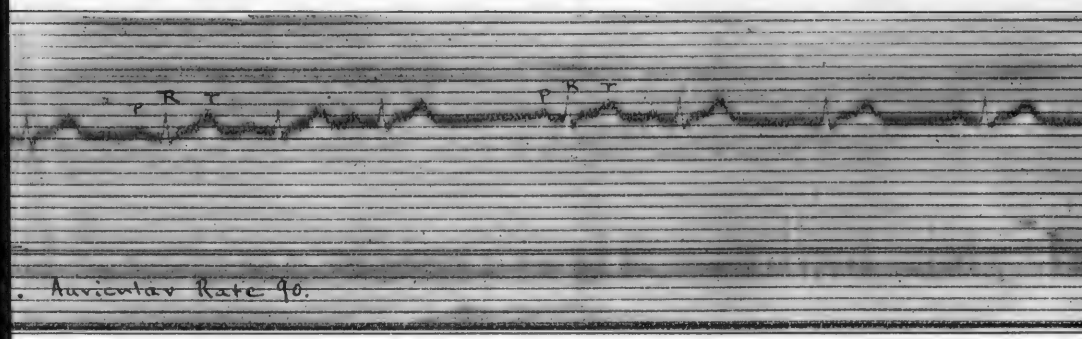
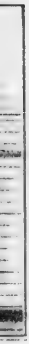
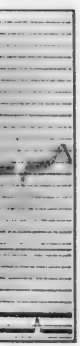
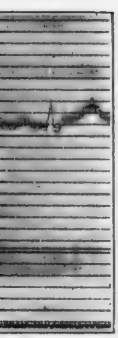


FIG. 7.

PLATE 35.







G. Canby Robinson.

PLATE 36.

FIG. 8. Curve 122.2. Case 2.
FIG. 9. Curve 179.2. Case 4.
FIG. 10. Curve 179.7. Case 4.
FIG. 11. Curve 179.23. Case 4.

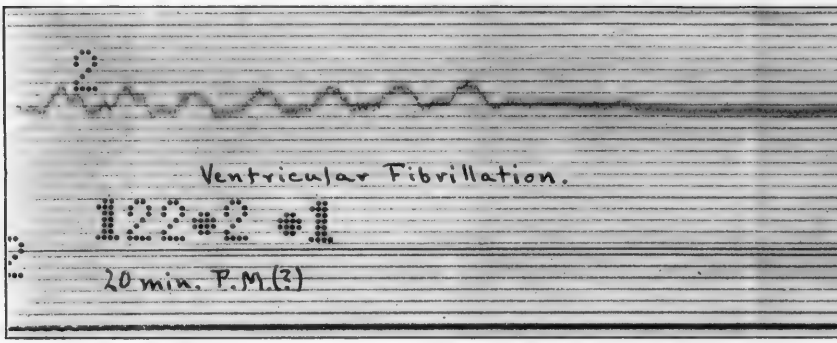
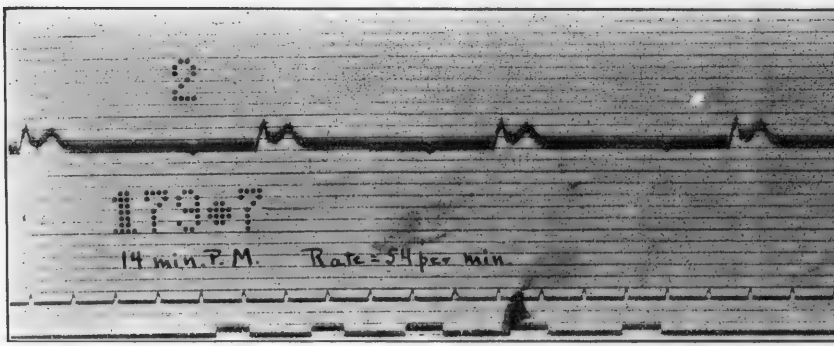
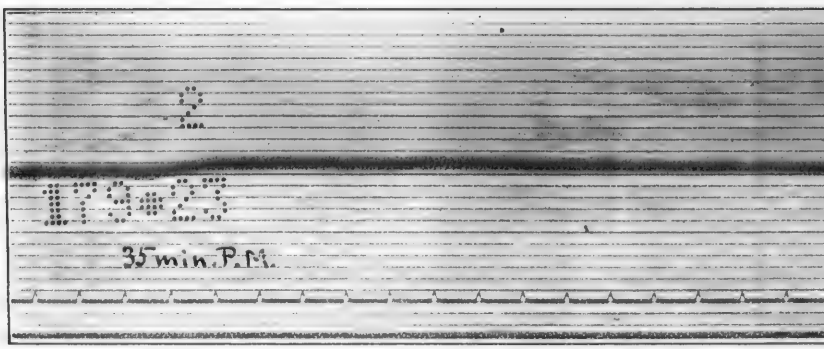

Ventricular Fibrillation.
20 min. P.M.(?)

FIG. 8.


14 min. P.M. Rate = 54 per min.


35 min. P.M.

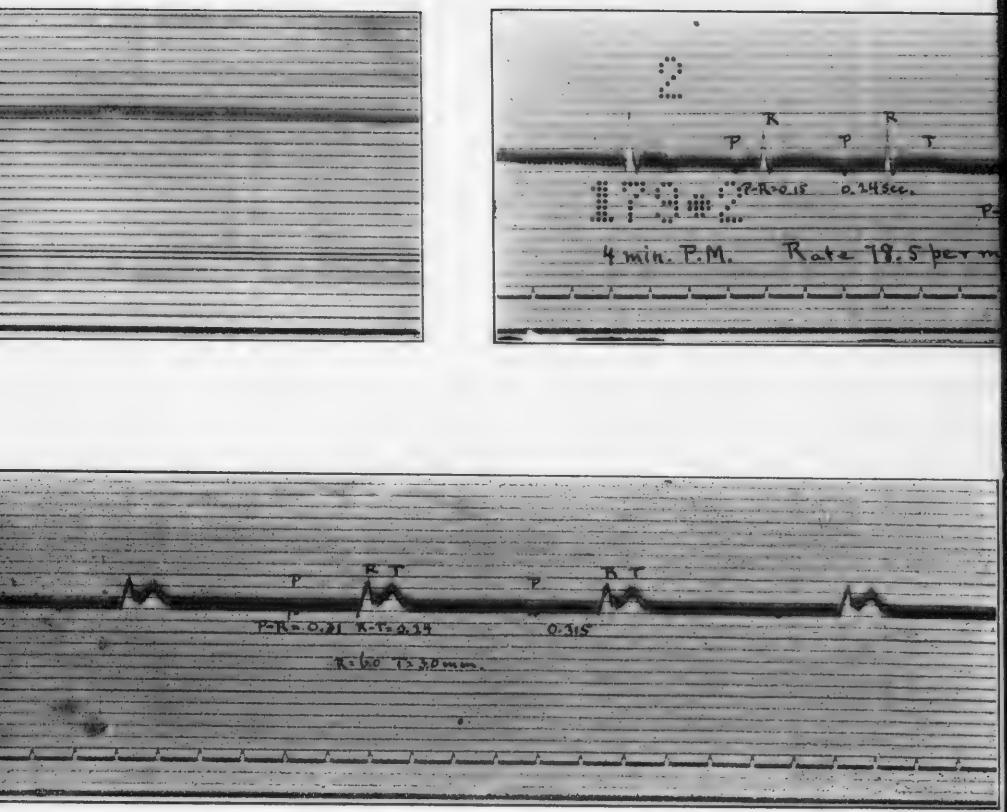

P
R
P
R
T
P-R=0.18
0.24sec.
4 min. P.M.
Rate 79.5 per m
P
R T
P
R T
P-R=0.21
R-T=0.34
0.315
R=60 T=30 mm.

FIG. 10.

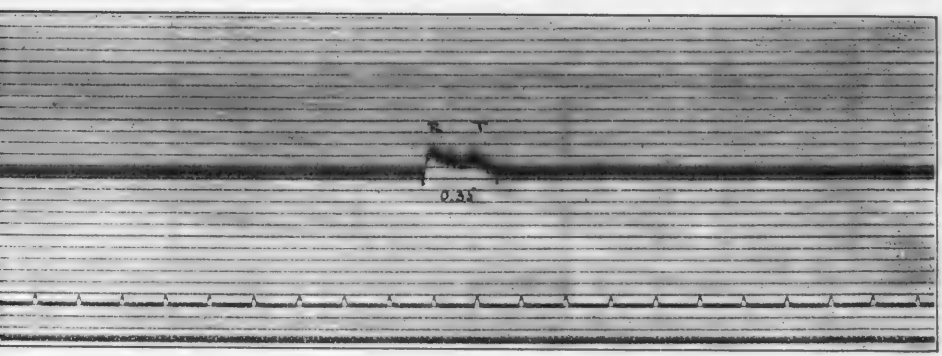

R
T
0.35

FIG. 11.

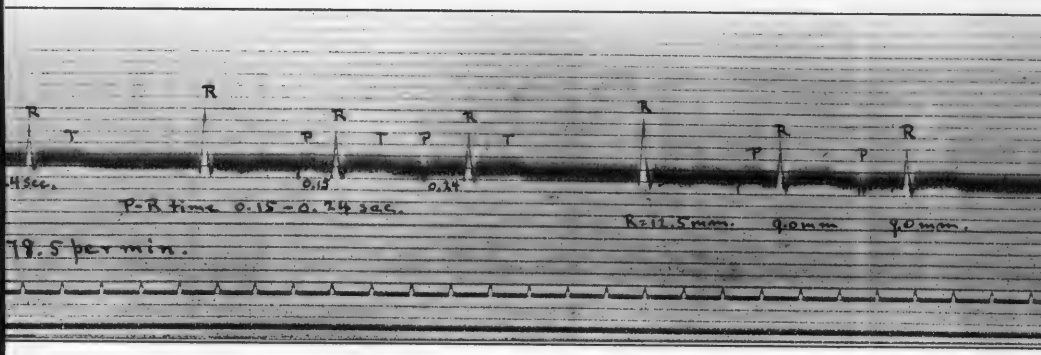

R
T
R
P
T
P
T
R
P
T
R
P
R
P
R
4sec.
P-R time 0.15 - 0.24 sec.
0.15
0.24
78.5 per min.
R=12.5mm. 9.0mm 9.0mm.

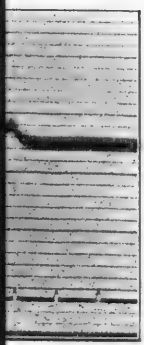
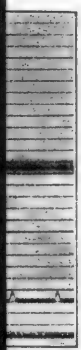
FIG. 9.



PLATE 36.

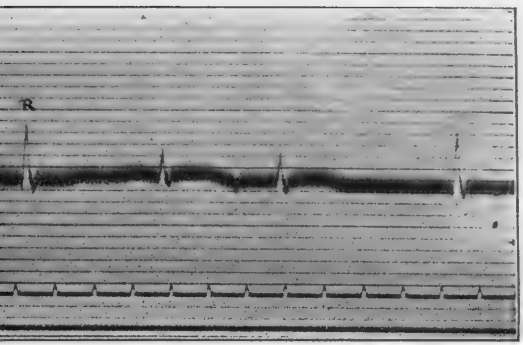


PLATE 37.

FIG. 12. Curve 149.2. Case 5.
FIG. 13. Curve 149.6. Case 5.
FIG. 14. Curve 149.15. Case 5.
FIG. 15. Curve 195.2. Case 6.
FIG. 16. Curve 195.5. Case 6.
FIG. 17. Curve 195.7. Case 6.

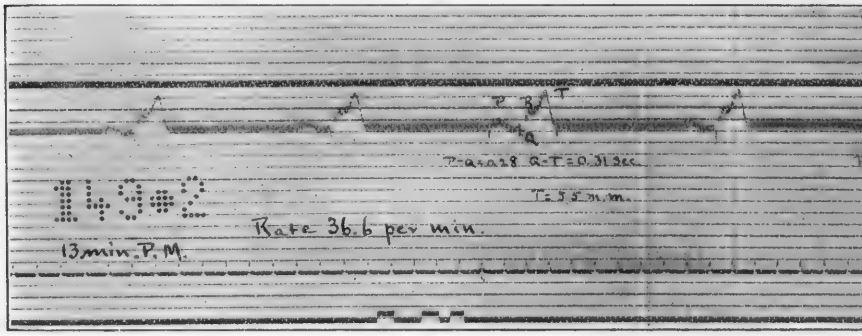
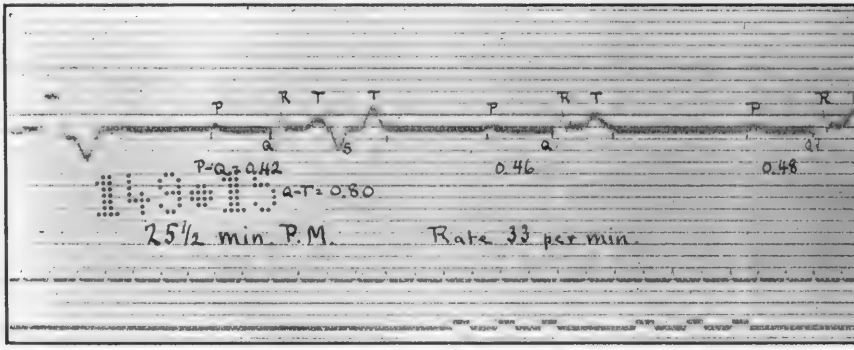

P
R
T
Q
P-Q=0.28 Q-T=0.31 sec.
T=5.5 m.m.
Rate 36.6 per min.
13 min. P.M.

FIG. 12.


P
R
T
Q
S
P-Q=0.42
Q-T=0.80
0.46
0.48
25½ min. P.M.
Rate 33 per min.

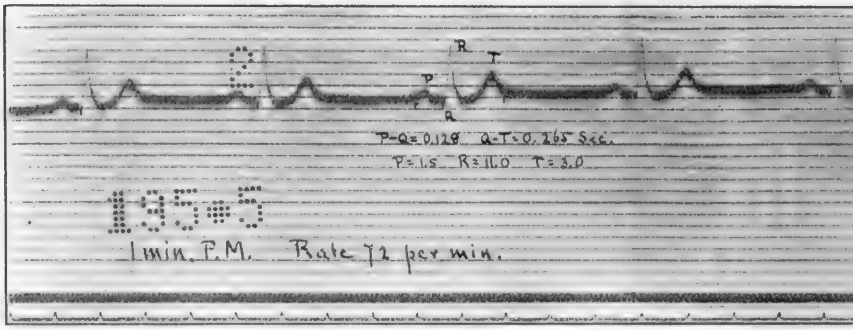

R
T
P
Q
P-Q=0.128 Q-T=0.265 sec.
P=1.5 R=11.0 T=3.0
1 min. P.M.
Rate 72 per min.

FIG. 16.

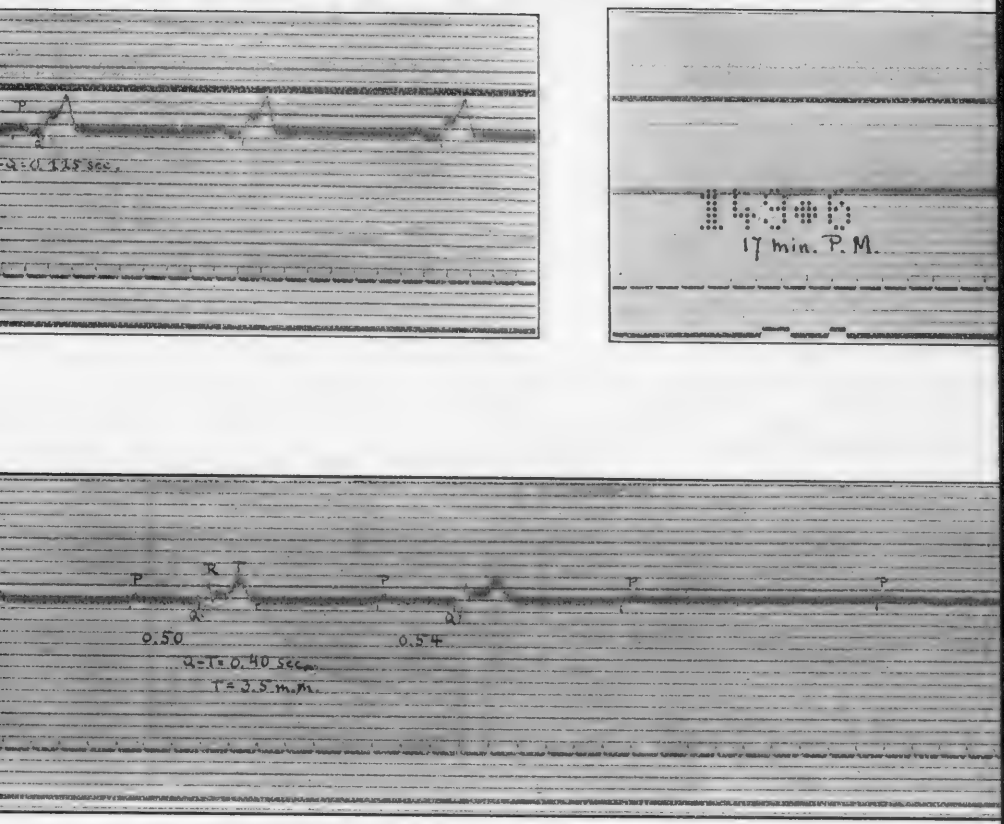
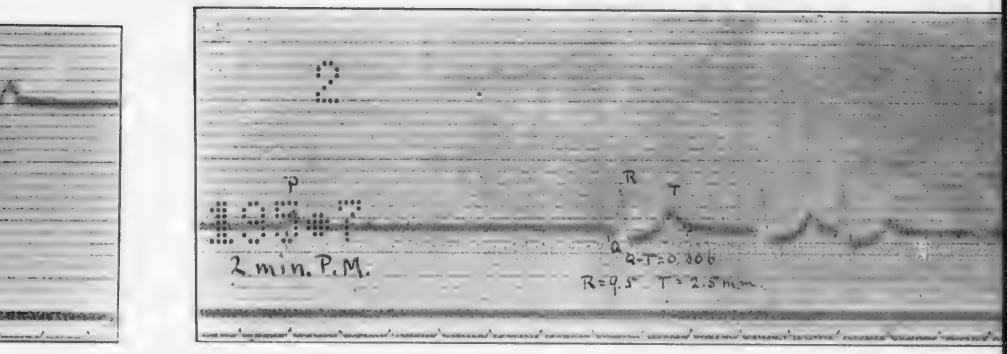

P
Q=0.125 sec.
4.30
17 min. P.M.
P
R
T
P
P
P
0.50
0.54
Q-T=0.40 sec.
T=2.5 m.m.

FIG. 14.


P
R
T
2 min. P.M.
Q-T=0.406
R=9.5 T=2.5 m.m.

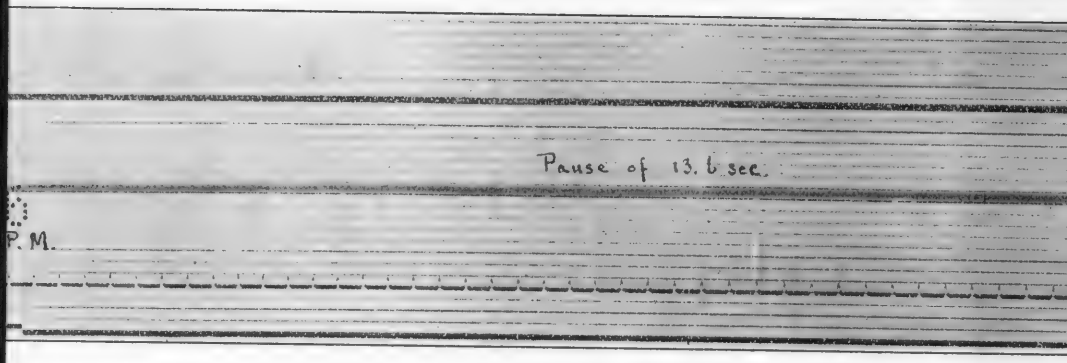
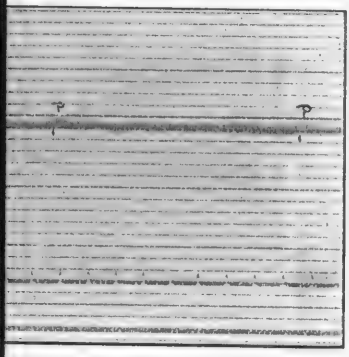


FIG. 13.



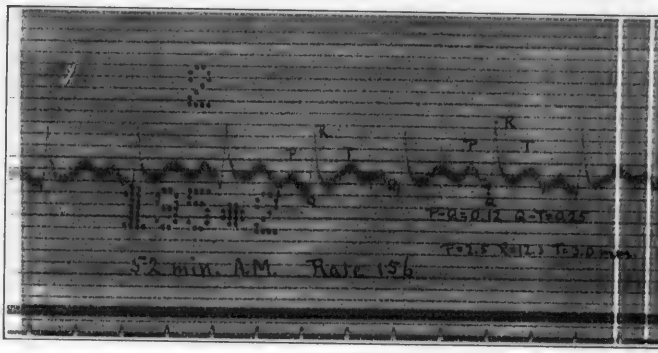


FIG. 15.

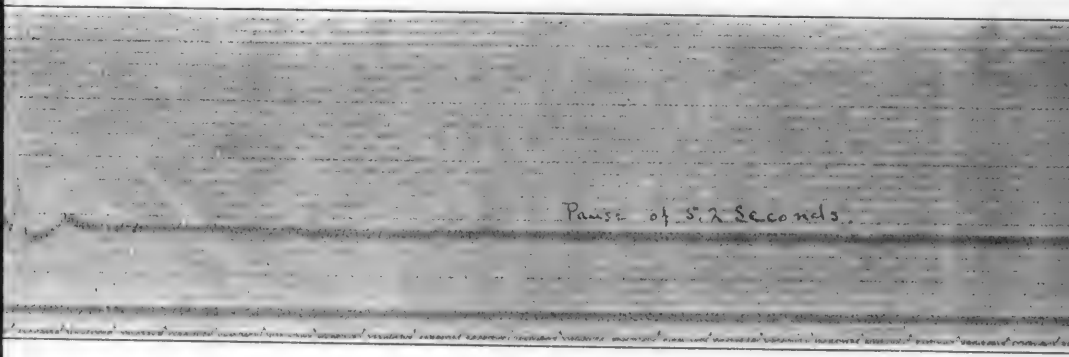
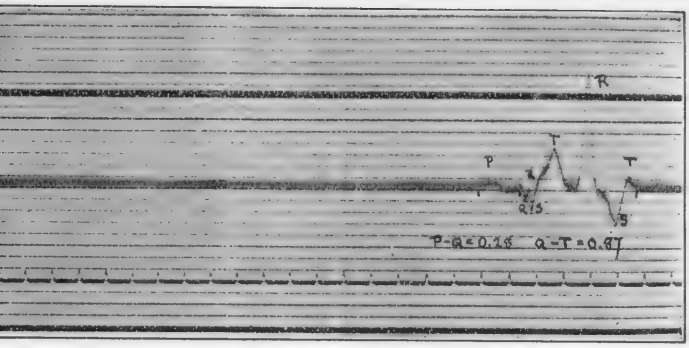
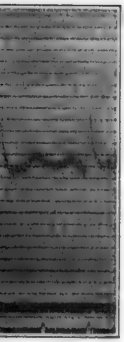
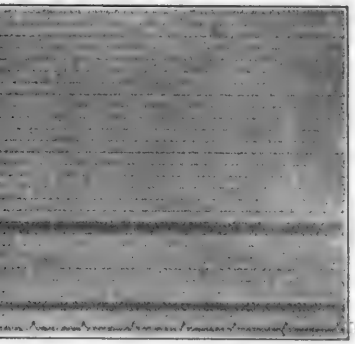


FIG. 17.


R
P
Q
S
T
P-Q=0.16 Q-T=0.47

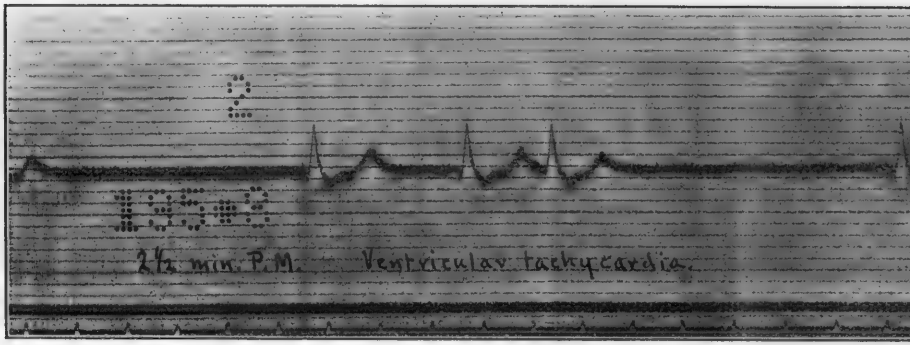




G. Canby Robinson.

PLATE 38.

FIG. 18. Curve 195.8. Case 6.
FIG. 19. Curve 195.12. Case 6.
FIG. 20. Curve 195.14. Case 6.


2½ min. P.M. Ventricular tachycardia.

Fig

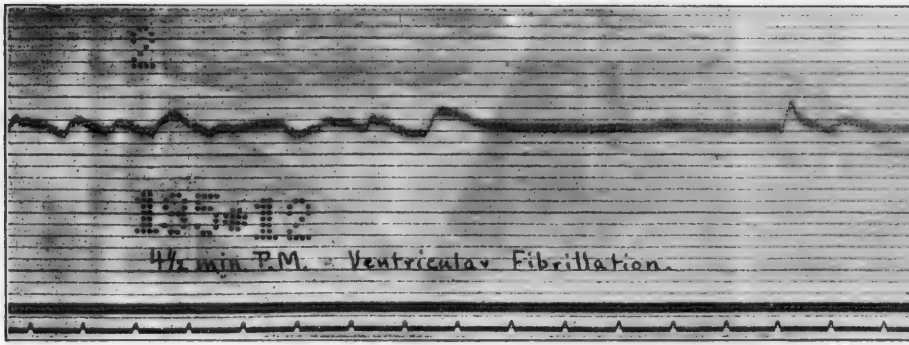
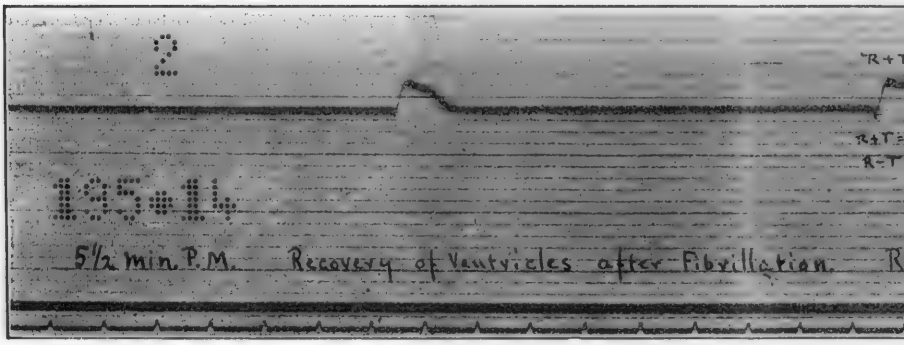
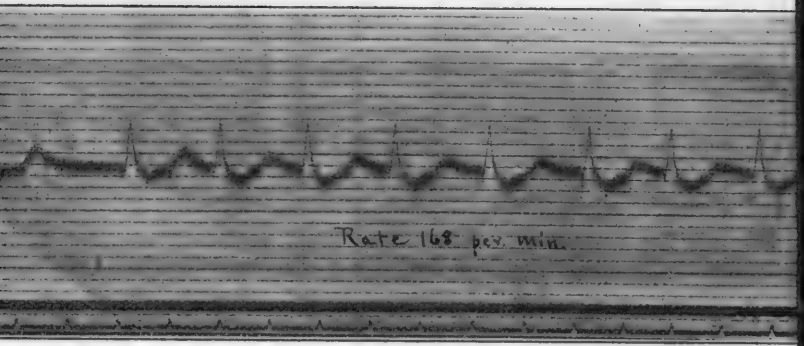
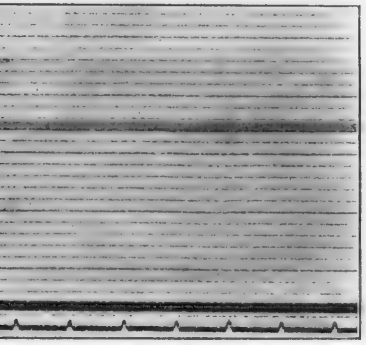

4½ min P.M. = Ventricular Fibrillation.

Fig. 19.


5½ min. P.M. Recovery of Ventricles after Fibrillation.



18.



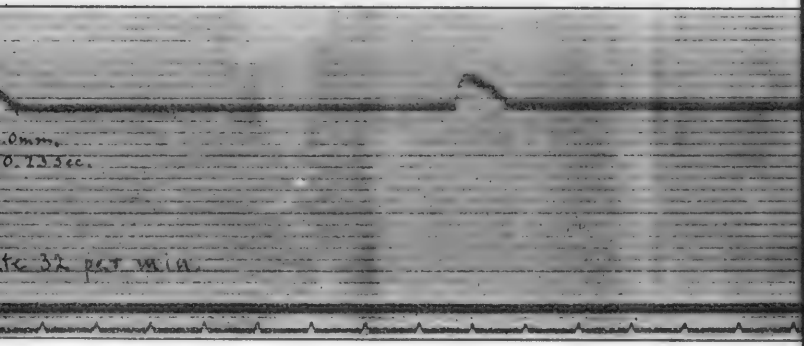


FIG. 20.

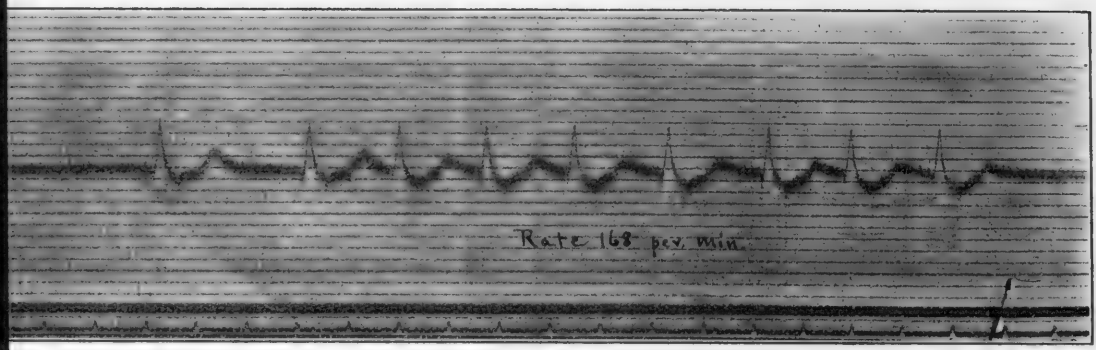
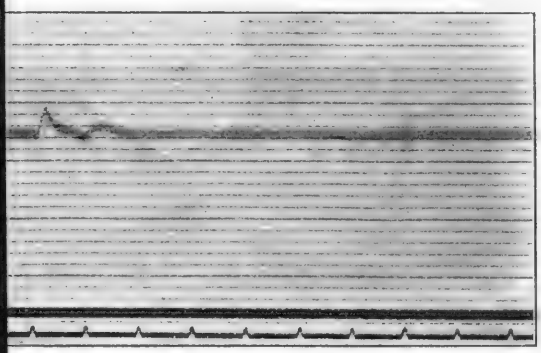


FIG. 18.



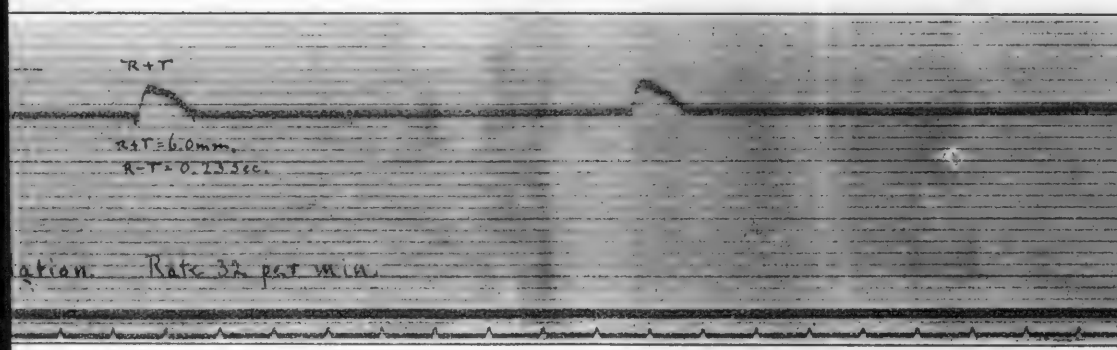
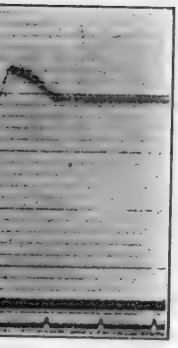


FIG. 20.

PLATE 38.



NITROGEN AND NUCLEIN METABOLISM IN GOUT.*

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For more than a century the pathology of gout has been associated with disturbances of uric acid metabolism. When the first theories of gout were advanced, there existed very little exact knowledge of the chemistry of uric acid and still less of its physiology. Every step in the progress of our knowledge of the chemistry of purin bodies, and of the biology of nuclein derivatives, led to a revision of the older theories of gout. A critical review of the most important views on this pathological condition has been published by Brugsch and Schittenhelm[1] and need not be repeated in this place.

The work of these authors was done in the light of the latest achievements in the biochemistry of nuclein compounds, and as a result chiefly of their own experiments they proposed a new theory to explain the disease. The principal advance made by these investigators is due to the fact that they placed their patients on a purin-free diet, and owing to this they were able to differentiate the process of endogenous from that of exogenous nuclein metabolism. They arrived at a conclusion similar to that of older writers; namely, that the pathology of gout is closely associated with that of nuclein metabolism. According to their view the disease is characterized by three conditions: first, retarded uric acid formation; second, retarded uric acid disintegration; and finally, retarded uric acid elimination.

The considerations which led them to formulate their theory were the following: the uric acid output of a gouty individual on a purin-free diet was lower than the output of a normal man under the same conditions. On the other hand, in the course of the disease the average uric acid content of the blood was higher than in health. If, however, purin bases, or nuclein material, were added to the purin-free diet, the increase in the elimination of uric acid of the gouty individual was lower in quantity, and at the same time more protracted than in health. A normal individual, on a purin-free diet after the intake of nuclein material, responded with a prompt increase in the output of uric acid, but this increased output had a very short duration.

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[1] Brugsch, T. B., and Schittenhelm, A. S., Der Nukleinstoffwechsel und seine Störungen, Jena, 1910.

This difference in the reaction of the organism to ingested purin suggested to Brugsch and Schittenhelm the view that the process of uric acid formation in the gouty organism was retarded as compared with that in normal individuals. Nevertheless, the absolute quantity of uric acid destroyed by the patient was higher than that destroyed by the normal individual, for the reason that at any given time the tissues of the patient contained more uric acid, and although its destruction at a given place was progressing with a lower intensity, more uric acid was destroyed in the body as a whole in a given period of time. The fact that the tissues and the blood of gouty individuals do not contain more uric acid than is generally found in them is the result of this arrangement.

Although the views of Brugsch and Schittenhelm met with some opposition, still in the main they were accepted. However, these authors neglected to include in the sphere of their observation the course of elimination of nitrogenous substances of non-nuclein origin. Hence it is possible that the abnormalities which they found in connection with nuclein metabolism may exist as a general abnormality of metabolism of all nitrogenous substances. The present investigation was undertaken with a view of obtaining light on this point and also to extend the knowledge of the progress of nucleic acid disintegration in the course of gout.

In a previous study on chronic granular nephritis the authors[2] observed that in that disease all nitrogenous end-products of metabolism were retarded in their elimination when the daily intake contained more nitrogen than the diseased kidneys could conveniently eliminate. However, if the eliminating capacity of the kidneys was taken into consideration, and the nitrogen intake regulated on the basis of this capacity, then the character of elimination in nephritis had a course similar to that in health.

For this reason it seemed to us of importance to begin the study of metabolism in the following manner: first, to establish the normal eliminating capacity of the kidneys for nitrogenous end-products of metabolism; second, to place the patient on a diet not exceeding this eliminating capacity; third, to compare the normal progress of nitrogenous output with that obtained after administration of additional nitrogenous substances derived from nucleic acid, or from proteins.

[2] Levene, P. A., Kristeller, L., and Manson, D., *Jour. Exper. Med.*, 1909, xi, 825.

The observations recorded here were made on a patient in a very advanced stage of gout. Very large gouty deposits in nearly all joints prevented him from any active movements. The patient was at first placed on a daily diet containing about six grams of nitrogen. When the daily output became fairly constant (about five and one half grams), the experiments were begun. These consisted in the addition of urea to the normal diet. The urea was added to the first morning meal and the nitrogen output was followed in twenty-four hour periods, day by day. In a similar manner experiments were carried out with asparagin, plasmon, uric acid, and nucleic acid.

It was observed that after the administration of very simple nitrogenous substances, such as urea and asparagin, the patient did not remove the surplus nitrogen in the same manner as a normal individual, or even as the patient with granular nephritis who was placed on a low nitrogen intake. The increase in the nitrogen output was comparatively low during the first twenty-four hour period, and this slightly increased excretion usually continued for several days.

This suggested a comparison of the character of nitrogen elimination when the patient was placed on a daily diet containing about thirteen grams of nitrogen. On this diet, after administration of urea, the rise in the nitrogen output took place with greater rapidity and with greater intensity than on the low protein diet. Also the nitrogen output in the intervals between the experiments appeared to be more uniform than when the patient had been on a low protein intake.

For this reason it was decided to perform a new series of experiments, placing the patient on a diet abundant in nitrogen. But even under these conditions the increase in the output of nitrogen, after the administration of additional nitrogenous substances, was very low and protracted. This made it difficult to detect the differences in the output during the intervals between experiments and during the days of the experiments.

A new series of experiments was then performed, in the course of which the additional nitrogenous products were added, not during one day, but during three or four consecutive days, and the output of nitrogen was followed in twenty-four hour periods for a

number of days. But this change in daily nitrogen intake brought little change in the results of the experiments, for the increase in nitrogen output after the administration of nitrogenous substances was rather insignificant, and the excretion was very often protracted. For the sake of convenience, therefore, it was decided to estimate the increase in the nitrogen output during the days of the experiment and during the two days following the experiment.

While the patient was under observation, which was for three winter terms, Medigreceanu and one of us[3] noticed that after the administration of purin bodies, the nitrogen output in animals had a more regular course if they received simultaneously considerable quantities of sodium bicarbonate. It was, therefore, decided to repeat the experiment on our patient, administering to him daily fifteen grams of this salt. It seemed that under these conditions the patient removed the surplus nitrogen intake with greater rapidity than without the bicarbonate of soda.

Together with the study of the character of the general nuclein metabolism an attempt was made to obtain information regarding the manner in which nucleic acid underwent disintegration in the organism of the gouty individual, with a view to ascertaining whether or not there existed any distinction in this regard between the gouty and the healthy organism.

It has been established in recent years that nucleic acids are composed of simple nucleotides, which in their turn consist of phosphoric acid and nucleosides. It has also been established recently that in the normal organism nucleic acid undergoes gradual decomposition, first into nucleotides, then into phosphoric acid and nucleosides, and finally into a base and a carbohydrate. Hence, it was planned to ascertain whether the complexity of the substance in which the purin bodies were administered to the organism had an influence on the rate and duration of the excessive nitrogen output. The patient received uric acid, hypoxanthine, inosine, guanosine, and nucleic acid in different experiments. For convenience of the analysis of the results of the experiments, the figures of the surplus output of nitrogen during the first twenty-four hours are presented in table I.

[3] Levene, P. A., and Medigreceanu, F., *Am. Jour. Physiol.*, 1911, xxvii, 438.

TABLE I.

Experiments.	N of standard diet in gm.	N of additional substance in gm.	Per cent. of N of additional substance eliminated in the first twenty-four hours.
First series.....	7.0	2.5 (urea)	65.0
	7.0	2.5 (asparagin)	59.0
	7.0	2.5 (uric acid)	26.0
	7.0	2.5 (uric acid)	29.0
	7.0	1.85 (nucleic acid)	50.0
Second series.....	6.0	2.0 (urea)	0
	10.0	2.0 (urea)	57.0
	13.0	2.0 (urea)	70.0
	13.0	2.0 (nucleic acid)	34.0
	13.0	2.0 (uric acid)	0
	13.0	2.0 (uric acid)	29.0
Third series.....	6.0	2.0 (urea)	0
	6.0	2.0 (urea)	17.5
	13.0	2.0 (urea)	69.0
	13.0	2.0 (hypoxanthine)	50.0
	13.0	2.0 (hypoxanthine)	15.5
	13.0	1.1 (inosine)	60.0
	13.0	1.5 (guanosine)	23.0
	13.0	2.0 (uric acid)	40.0

The figures presented in the table do not show a great regularity in the removal of the surplus nitrogen ingested. It is clearly seen, however, that the removal of nitrogen ingested in the form of nucleic acid derivatives is lagging behind the nitrogen output after the administration of urea. The nearest to urea in its behavior is inosine, then follow nucleic acid, hypoxanthine, and the most imperfect elimination follows the administration of uric acid and of guanosine.

The rate of elimination of the surplus nitrogen is controlled by two factors, the rate of absorption and that of oxidation, and it is possible that the low figures observed after uric acid and guanosine intake are brought about by the comparative insolubility of these substances. Both inosine and hypoxanthine possess a comparatively high degree of solubility, and it appears as if the elimination of inosine nitrogen proceeds with greater rapidity.

It is not improbable that the factors of purin metabolism discovered by Walter Jones come into play in this instance. Jones[4] discovered that oxidation of the nucleosides and of purin bases is brought about by different enzymes, and it is possible that in this instance the nucleoside-oxidases were more active than the purin-

[4] Jones, W., *Jour. Biol. Chem.*, 1911, ix, 129, 169.

oxidases. This may also explain the reason for the more rapid nitrogen elimination after administration of nucleic acids. As a general rule, however, we noticed in our patient a comparatively slow rate of oxidation of nucleic acid derivatives, particularly of uric acid.

However, a review of the figures recording the surplus nitrogen output for the entire period of the experiments indicates that in the course of some days the greatest part of the nuclein nitrogen is removed from the body, although again in this respect there is noted a considerable irregularity.

The principal form in which the nitrogenous products of nucleic acid finally reappear in the urine seems to be urea. The increase in uric acid output and in the output of purin bodies is rather small, and the highest increase was noted after the administration of nucleic acid.

CONCLUSIONS.

Thus, our conclusions harmonize with those of Brugsch and Schittenhelm in so far as they are concerned with the nuclein metabolism. They add, however, the observation that the elimination of nitrogenous substances of protein origin has a protracted character, and that the oxidation of as simple a substance as asparagin proceeds at a subnormal rate.

The most striking peculiarity observed in our patient was the very imperfect elimination of ingested urea when the patient was placed on a diet containing only six grams of nitrogen per day, and a much more complete elimination when the diet contained thirteen grams of nitrogen.

EXPERIMENTAL PART.

Methods of Analysis.—Total nitrogen was estimated by the Kjeldahl-Gunning method; urea, originally by Folin's method, and later by the Levene and Meyer[5] modification of Benedict's process; ammonia, by the method of Shafer-Folin; uric acid, by the method of Ludwig-Salkowsky. Purin bases were estimated by treating the mother liquor from uric acid with mercuric sulphate, decomposing the precipitate, and reprecipitating the purin bases with ammoniacal silver solution.

Food Analysis.—All cereals were obtained in large quantities and a sample of each new supply was analyzed. Milk, cream, butter, eggs, and bread were analyzed from time to time. Vegetables were not analyzed, their composition being calculated on the basis of figures given by Koenig.

[5] Levene, P. A., and Meyer, G. M., *Jour. Am. Chem. Soc.*, 1909, xxxi, 717.

Diet.—The diet all through the experiments was purin-free. The patient was in charge of a special nurse who was familiar with the diet problem, and all meals were prepared by her personally. The diet consisted of eggs, milk, cream, butter, bread, cereals, vegetables, and fruit.

History.—The patient was Allen S., 44 years of age, a native of England. He had suffered from gout for twenty-seven years, and in the early period of the disease had frequent acute attacks. All his joints were deformed and stiff, and had been so since he had been under our observation.

The metabolism studies were carried out during the winter months of three consecutive years. With the beginning of warm weather the daily variations in nitrogen output were so great that the investigations had to be interrupted. Also in the course of the winter it generally required many weeks of a given diet before the nitrogen elimination approached a condition of equilibrium.

FIRST SERIES OF EXPERIMENTS.

During this series of experiments the additional substance was administered in one day periods and the nitrogen output was followed for several days after the administration. The standard output was calculated on the basis of an observation lasting eight days, from April 9 to April 16. The average output of total nitrogen was 6.72 gm. per day; of urea nitrogen, 5.68 gm., or 84.5 per cent. of the total; and of uric acid nitrogen, 0.081 gm., or 1.2 per cent. of the total.

The eliminating capacity of the kidney was determined by administration of urea. Two and one half gm. of nitrogen in the form of urea were given on April 26, 1909. During that day there were eliminated in excess over the standard days 1.63 gm. of nitrogen, and in the course of the three days that followed the administration, there were eliminated 3.67 gm. of nitrogen. The increase in urea nitrogen output for the same interval was 3.13 gm. and the average uric acid output per day was 0.078 gm. In previous observations on men and normal animals it was demonstrated that all of the urea administered is eliminated within the first twenty-four hours. In the present experiment during the same period only 65 per cent. of the excessive nitrogen intake was removed. Moreover, part of the excessive elimination was due to the diuretic effect of the substance. By this is explained the fact that of the excessive nitrogen output only 85 per cent. was in the form of urea. The daily output of uric acid for the same period did not differ materially from the normal.

Substances other than urea tested during this series were asparagin, uric acid, and nucleic acid.

Asparagin was given April 17, 1909. The additional nitrogen intake was 2.5 gm. The excessive output for forty-eight hours was 2.23 gm., the excessive output of urea nitrogen for the same period was 2.58 gm., and the daily output of uric acid nitrogen was 0.014 gm., which is not very significant. In previous observations on men and on normal animals it was found that the nitrogen of asparagin was removed with the same rapidity as urea; namely, all in the course of the first twenty-four hours. In this instance only 59 per cent. of the excessive intake was removed during that period.

Uric Acid was administered in two experiments. In the first experiment 2.5 gm. of nitrogen were given on May 6, 1909. During the following four days

there were eliminated 3.12 gm. of total nitrogen, 2.97 gm. of urea nitrogen, and 0.050 gm. of uric acid nitrogen in excess over the normal. The significant feature of this experiment is the fact that in the first twenty-four hours only 0.26 gm. of excessive nitrogen was removed, or 10 per cent. of the additional intake.

The second uric acid experiment was performed on May 10, 1909. The same quantity of uric acid was given. The excessive nitrogen output in the four days that followed was 3.23 gm., the excessive urea nitrogen was 3.63 gm., and the uric acid output 0.078 gm. per day, practically the same as on standard days. Again the significant feature of this experiment was the low nitrogen output in the first twenty-four hours; namely, only 29 per cent. of the excessive intake.

Nucleic Acid was administered on May 19, 1909. There were given 1.83 gm. of nitrogen in the form of nucleic acid. Following the second uric acid experiment the daily nitrogen output never came down to that of the original standard period. Therefore, the elimination after nucleic acid intake was compared, not with the first standard period, but with the average output of the five days preceding the nucleic acid intake. The daily average output for those five days was: total nitrogen 7.55 gm., urea nitrogen 6.61 gm., or 87.5 per cent. of the total, and uric acid nitrogen 0.0758 gm., or 1 per cent. of the total.

During the four days following the administration of nucleic acid there was an excessive output of 2.98 gm. of total nitrogen, 2.50 gm. of urea, and the uric acid nitrogen showed an increase of 0.0783 gm. It is noteworthy that in the first twenty-four hours the increase in the nitrogen output was over 50 per cent. of the excessive intake. Of course in part the increased nitrogen output during this period was occasioned by the diuresis produced by the absorption of nucleic acid.

SECOND SERIES OF EXPERIMENTS.

This series of observations lasted from November, 1909, to March, 1910. Again on this occasion an attempt was made to ascertain the most favorable conditions for the elimination of the nitrogen of the substances added in excess of the standard diet. The patient was placed in a condition approaching nitrogenous equilibrium on a diet containing less than 6 gm. of nitrogen. To this diet on the day of the experiment 2 gm. of nitrogen in the form of urea were added, but the nitrogen elimination remained unaffected by it. The nitrogen intake was then raised to 10 gm., and after the patient was placed in a condition of nitrogenous equilibrium, 2 gm. of nitrogen in the form of urea were added. But even then the increase in nitrogen elimination was very slow, and in one experiment the total increase in nitrogen output after an additional administration of 2 gm. of nitrogen was not much above 50 per cent. over the normal. The nitrogen intake was raised to 13 gm. per day and the urea administration proved satisfactory. Experiments were then performed with uric acid and with nucleic acid.

Experiments with an Intake of Six Grams of Nitrogen.—The normal output previous to the administration of urea was 5.98 gm. After the administration of an additional 2 gm. of nitrogen in the form of urea, on November 10, the nitrogen output was scarcely changed, being on the day of the experiment 6.13 gm. and on the day following 5.74 gm. The urea and uric acid output also remained little

changed. After this experiment the normal nitrogen output fell to 5.22 gm. as the average of seven days. The average urea output for the same days was 4.06 gm. Another experiment with the administration of 2 gm. of nitrogen in the form of urea was made. The nitrogen and urea output after this administration remained practically unchanged. Thus, on the low nitrogen intake there was a retention of nitrogen even when it was administered in the form of urea.

Experiments with an Intake of Ten Grams of Nitrogen.—On a diet of 10 gm. of nitrogen the average daily output for six days was 8.82 gm. For the same period the urea output was 7.51 gm. per day, or 85.2 per cent. of the total nitrogen. The uric acid output was 0.0869 gm. per day and the nitrogen of the purin bases was 0.062 gm. per day.

Urea Experiments.—An experiment with the administration of an additional 2 gm. of nitrogen in the form of urea was made on December 14. During the first twenty-four hours after the administration there was an excessive output of nitrogen of 1.15 gm., or 57 per cent. of the total excessive intake. There was, however, a protracted increase in the output for four days, so that in this experiment the total increase in the output exceeded the intake, although the diuresis produced by the substance was insignificant.

On December 21 another experiment was performed with the administration of 2 gm. of nitrogen in the form of urea. During the first twenty-four hours of the experiment 0.36 gm. of nitrogen was removed, or 18 per cent. of the intake. The following day there was an increase of the nitrogen output of 0.59 gm., or 30 per cent. of the intake, and on the third and fourth days there was still a slight increase. The total increased output for four days exceeded the normal by 59 per cent. of the excessive intake.

Experiments with an Intake of Thirteen Grams of Nitrogen.—The nitrogen intake was again raised to 13 gm. per day and the additional substances were given on four successive days. The output was then followed for about eight days after the beginning of the administration of the additional substances. The average output for five days was 12.20 gm. The first experiment began on January 19. For four successive days 2 gm. of nitrogen in the form of urea were administered. During the first twenty-four hours 1.41 gm. of excessive nitrogen were removed, or 70 per cent. of the intake. The following day again 1.40 gm. of nitrogen were removed. In course of eight days from the beginning of the experiment 8.94 gm. of nitrogen were removed, of which 6.68 gm. were in the form of urea, 0.0751 gm. in the form of uric acid, and 0.0074 gm. in the form of purin bases.

It is seen from these figures that the elimination of the excessive nitrogen was most favorable on this diet and, therefore, experiments were performed with nucleic acid and with uric acid. The nucleic acid experiments began on January 31, 1910.

Nucleic Acid Experiments.—Two gm. of nitrogen in the form of nucleic acid were given on four successive days and the nitrogen output was followed for eight days after the beginning of the experiment. In the first twenty-four hours there was eliminated 0.68 gm. of nitrogen in excess of the normal output, and each following day the excessive nitrogen output increased in value reach-

ing on the fifth day 2 gm. The total excessive output for eight days was 8.09 gm. of nitrogen of which 5.91 gm. were in the form of urea, 0.500 gm. in that of uric acid, and 0.415 gm. in the form of purin bases.

Uric Acid Experiments.—On February 15, experiments with uric acid were begun. The experiments lasted four days. On each day the patient received 2 gm. of nitrogen in the form of uric acid. During the first twenty-four hours the nitrogen output remained unchanged. In the second twenty-four hours, 0.82 gm. of excessive nitrogen was removed; on the following day 1.50 gm., and in the course of nine days 7.15 gm. of nitrogen were removed, of which 5.22 gm. were in the form of urea, 0.381 gm. in the form of uric acid.

On March 7, the second experiment with uric acid was performed. Two gm. of nitrogen in the form of uric acid were given daily on four successive days. During the first twenty-four hours 0.59 gm. of excessive nitrogen was removed, on the second day 1.34 gm., and on the fifth day the nitrogen output came down to normal. In the course of the five days the total excessive nitrogen output was 5.39 gm., of which 5.17 gm. were in the form of urea, and 0.187 gm. in the form of uric acid.

It is seen from these figures that on a high nitrogen intake the removal of excessive nitrogen is more complete than on a low protein intake. But it is not so complete as in health, so that even urea is in part retained in the organism. The nitrogen of nucleic acid and uric acid is removed at a slower rate than that of urea. This, of course, occurs also in health. Comparing the character of nitrogen elimination after the administration of nucleic acid and of uric acid one notes that after uric acid administration the nitrogen output proceeded at a lower rate of speed, and was less complete. This is observed also in health. The increase in the uric acid output was 6.2 per cent. of the total in the nucleic acid period, and 5.3 and 3.5 per cent. respectively in the two uric acid periods.

THIRD SERIES OF EXPERIMENTS.

This series lasted from November, 1910, to April, 1911. In this series of experiments it was again aimed to ascertain whether the quantity of nitrogen in the standard diet influenced the rate of elimination of excessive nitrogen added to the standard diet. The patient was again placed on a diet containing about 6 gm. of nitrogen. He came to a condition approaching nitrogenous equilibrium when the average daily output for five days was 6.29 gm. of nitrogen.

Urea Experiments.—On December 3 an experiment was begun with the administration of urea. Two gm. of nitrogen in the form of urea were given on three consecutive days. There was no increase in the nitrogen output during the first twenty-four hours. During the second twenty-four hours the increase of nitrogen output was 0.076 gm., and on the third day 0.072 gm. After this the output came down to normal. In December the daily nitrogen output on

the standard diet was lower, reaching an average of 5 gm. per day. On December 13 an experiment was begun with the administration of 2 gm. of urea per day on three consecutive days. The excessive output for the first day was 0.35 gm., the second day 1.30 gm., the third day 1.65 gm., and for five days following the beginning of the experiment 4.54 gm. of nitrogen were removed.

Again it follows from these observations that on a low nitrogen diet the elimination of the excessive nitrogen was very incomplete. Hence the diet was changed so as to contain 13 grams of nitrogen per day. On this diet the daily output was 10.50 grams of nitrogen per day.

On January 23 an experiment was performed with urea. Two gm. of urea were administered per day on three consecutive days. The excessive output during the first twenty-four hours was 1.38 gm., or 69 per cent. of the intake. The following day the output was 2.36 gm., and in five days after the beginning of the experiment the excessive nitrogen output was 6.31 gm.

It is seen from these figures that again on this occasion the increase in the nitrogen intake brought about a condition which permitted the nitrogen added in the form of urea to be removed more completely and in a shorter period of time. Experiments were then performed with hypoxanthine, inosine, guanosine, and uric acid.

Hypoxanthine Experiments.—1. Two gm. of nitrogen in the form of hypoxanthine were given on three successive days beginning January 30. On the first day of the experiment 1.02 gm. of excessive nitrogen were removed, and in five days following the beginning of the experiment there were removed 4.17 gm. of nitrogen in excess over the normal, or 69.5 per cent.

2. The second experiment with hypoxanthine was performed on February 13, when 2 gm. of nitrogen in the form of hypoxanthine were given daily for three days. During the first twenty-four hours there was an excessive elimination of 0.23 gm. of nitrogen, the second day 0.84 gm. of nitrogen, and in the course of six days from the beginning of the experiment 4.47 gm. of nitrogen were removed, or 74.5 per cent. of the excessive intake.

The daily output of nitrogen on the standard diet changed at about this period, reaching 10.85 grams per day.

Inosine Experiments.—One gm. of nitrogen in the form of inosine was given on three consecutive days beginning February 24. The excessive nitrogen output on the first day of the experiment was 0.65 gm. of nitrogen, and in three days of the experiment there were removed 2 gm. of excessive nitrogen, or 60 per cent. of the excessive intake.

The nitrogen output on the standard diet changed again, giving an average of about 10.17 grams of nitrogen.

Uric Acid Experiments.—Two gm. of nitrogen in the form of uric acid were given on three consecutive days beginning March 6. The excessive output during the first twenty-four hours of the experiment was 0.81 gm., on the second day 1.31 gm., and in five days after the beginning of the experiment there were eliminated 6.43 gm. of excessive nitrogen, or 100 per cent. of the excessive intake.

After this period the condition of the patient was such that the physician deemed it advisable to prescribe diuretin for him. The daily output on the normal diet then rose to 11.96 grams per day.

Guanosine Experiments.—One and a half gm. of nitrogen in form of guanosine were given in two days beginning April 7. In the first twenty-four hours there was an excessive nitrogen output of 0.46 gm.; in three days after the beginning of the experiment there was an increase of nitrogen output of 2.36 gm., or 78.6 per cent. of the total intake.

In order to facilitate the analysis of the behavior of the different derivatives of nucleic acid after their administration the figures of the excessive nitrogen elimination are presented in the following tables (tables II, III, and IV).

Date.	April, 1909.							
	9	10	11	12	13	14	15	16
Intake:								
Nitrogen in gm.	7.08	7.22	7.08	7.12	7.16	7.16	7.12	7.00
Water in c.c.	2,350.00	2,350.00	2,350.00	2,350.00	2,350.00	2,350.00	2,250.00	2,250.00
Calories	2,690.00	2,701.00	2,690.00	2,693.00	2,696.00	2,690.00	2,209.00	2,202.00
Additional N intake								
Output:								
Urine in c.c.	1,878.00	2,085.00	1,790.00	2,235.00	1,833.00	1,665.00	2,041.00	2,070.00
Urine specific gravity	1.009	1.010	1.009	1.010	1.011	1.009	1.010	1.009
Urine N in gm.	6.71	6.93	6.49	6.73	6.26	6.45	6.88	7.24
Feces N in gm.	1.74	2.45	1.65	0.49	1.70	0.83	1.11	1.76
Total N in gm.	8.45	9.38	8.14	7.22	7.96	7.28	7.99	9.00
Urea N in gm.	5.76	6.05	5.53	5.83	5.45	5.45	5.88	6.19
Per cent. of total urine N.	85.40	87.30	85.20	86.60	87.00	84.50	85.50	85.50
Ammonia N in gm.	0.15	0.18	0.24	0.13	0.17	0.18	0.22	0.24
Per cent. of total urine N.	3.30	2.60	3.90	2.00	2.70	2.80	3.00	3.40
Uric acid N in gm.	0.080	.....	0.0913	0.0837	0.087	0.083	0.089	0.0724
Per cent. of total urine N.	1.20	.....	1.40	1.30	1.39	1.30	1.30	1.00
Creatinin N in gm.	.....	.....	0.237	.....	.....	.....	0.257	.....
Creatin in gm.	.....	.....	0.018	.....	.....	.....	0.009	.....
Creatinin + creatin in gm.	.....	.....	0.255	.....	.....	.....	0.266	.....
Albumin gm. per 1,000 c.c.	0.3	0.2	0.3	0.3	0.2	0.4	0.3	0.3
Body weight in kilos.	83.46	83.46	83.46	83.68	83.91	83.68	83.91	83.80

TABLE II. *First Series.*

						May, 1909.					
16	17	18	26	27	28	6	7	8	9	10	11
7.00	6.92	7.10	7.08	7.20	7.04	7.06	6.96	7.18	7.00	7.02	6.88
2,250.00	2,250.00	2,250.00	2,450.00	2,250.00	2,250.00	2,250.00	2,250.00	2,250.00	2,250.00	2,250.00	2,250.00
2,202.00	2,200.00	2,214.00	2,270.00	2,222.00	2,210.00	2,270.00	2,200.00	2,220.00	2,210.00	2,210.00	2,200.00
.....	Asparagin 2.5 gm. N	.....	Urea 2.5 gm. N	.....	.....	Uric acid 2 gm. N	.....	.....	.....	Uric acid 2 gm. N	.....
2,070.00	2,230.00	1,705.00	2,650.00	2,250.00	2,570.00	1,767.00	1,995.00	2,375.00	1,820.00	2,165.00	2,310.00
1.009	1.010	1.008	1.008	1.010	1.007	1.009	1.008	1.007	1.010	1.008	1.009
7.24	8.19	7.48	8.35	7.83	7.65	6.98	7.62	7.90	7.50	7.44	7.88
1.76	1.35	1.27	1.76	1.16	1.10	1.08	1.27	0.74	1.93	1.33	1.37
9.00	9.54	8.75	10.11	8.99	8.75	8.06	8.89	8.64	9.43	8.77	9.25
6.19	7.30	6.64	7.20	6.39	6.58	6.16	6.58	6.90	6.05	6.03	7.17
85.50	89.10	90.00	86.20	81.60	86.00	90.0	86.30	87.00	80.60	81.00	91.00
0.24	0.21	0.19	0.27	0.23	0.21	0.17	0.18	0.10	0.16	0.26	0.19
3.40	2.60	2.60	3.20	3.00	2.90	2.20	2.30	1.40	2.20	3.50	2.50
0.0724	0.0988	0.0907	0.0736	0.0945	0.0648	0.0836	0.077	0.1617	0.0870	0.0813	0.077
1.00	1.21	1.20	0.86	1.21	0.85	1.21	1.02	2.06	1.16	1.08	1.00
.....	0.247	0.240	.....	.....	.....	.....	.....	.....	.....	.....	.....
.....	0.021	0.019	.....	.....	.....	.....	.....	.....	.....	.....	.....
.....	0.268	0.259	.....	.....	.....	.....	.....	.....	.....	.....	.....
0.3	0.3	0.3	0.3	0.3	0.3	.....	0.9	.....	0.5	0.5	0.3
83.80	83.91	83.57	83.68	83.46	83.68	82.72	82.78	82.78	82.66	83.23	83.12

12	13	14	15	16	17	18	19	20	21	22
6.96	6.94	7.00	7.06	7.06	7.00	7.12	7.06	7.10	7.24	7.10
2,250.00	2,250.00	2,250.00	2,250.00	2,250.00	2,250.00	2,250.00	2,250.00	2,250.00	2,250.00	2,250.00
2,200.00	2,200.00	2,210.00	2,210.00	2,210.00	2,210.00	2,210.00	2,210.00	2,210.00	2,220.00	2,210.00
.....	.....	.....	.....	.....	.....	.....	Nucleic acid 1.85 gm. N	.....	.....	.....
1,955.00	2,140.00	2,040.00	1,570.00	2,010.00	2,255.00	2,305.00	2,620.00	2,360.00	2,295.00	2,225.00
1.010	1.008	1.009	1.008	1.008	1.009	1.010	1.009	1.008	1.008	1.008
7.46	7.33	7.42	6.90	8.04	7.59	7.79	8.52	8.39	8.29	7.98
1.06	1.16	1.43	1.08	0.10	1.43	0.22	0.72	2.22	1.17	0.97
8.52	8.49	8.85	7.98	8.14	9.02	8.01	9.24	10.61	9.46	8.95
6.56	6.59	6.56	6.29	7.15	6.33	6.71	7.50	7.46	7.19	6.79
88.00	89.00	88.40	90.00	89.00	83.20	86.00	88.00	88.80	86.60	85.00
0.18	0.16	0.16	0.13	0.16	0.19	0.18	0.20	0.20	0.24	0.16
2.40	2.10	2.20	2.00	2.00	2.60	2.40	2.40	2.50	3.00	2.00
0.0853	0.0583	0.0837	0.0638	0.0860	0.0693	0.0764	0.1053	0.0944	0.1021	0.0797
1.14	0.80	1.13	1.00	1.07	0.92	1.00	1.24	1.13	1.35	1.00
.....	.....	.....	.....	.....	.....	0.266	0.281	0.259	.....	.....
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
0.3	0.3	0.2	.....	.....	0.3	0.3	0.4	0.3	0.3	0.3
83.12	83.00	83.23	83.00	83.12	83.34	83.23	83.34	83.23	83.12	83.12

Date.	November, 1909.							
	9	10	11	12	13	14	15	16
Intake:								
Nitrogen in gm.	5.62	5.66	5.64	5.66	5.58	5.60	5.62	5.72
Water in c.c.	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00
Calories	2,411.00	2,414.00	2,412.00	2,416.00	2,407.00	2,409.00	2,410.00	2,418.00
Additional N intake		Urea 2 gm. N						
Output:								
Urine in c.c.	1,860.00	1,965.00	1,930.00	1,450.00	1,550.00	1,600.00	1,600.00	1,690.00
Urine specific gravity	1.009	1.008	1.007	1.007	1.008	1.009	1.009	1.008
Urine N in gm.	5.98	6.13	5.74	5.06	5.21	5.25	5.06	5.16
Feces N in gm.	1.68	1.50	1.17	0.77	1.44	1.73	1.34	1.37
Total N in gm.	7.66	7.63	6.91	5.83	6.65	6.98	6.40	6.53
Urea N in gm.	4.72	5.12	4.56	3.96	4.06	4.17	4.08	4.08
Per cent. of total urine N.	79.60	83.50	79.80	78.30	78.00	79.40	80.60	79.70
Ammonia N in gm.	0.24	0.20	0.25	0.22	0.22	0.20	0.22	0.26
Per cent. of total urine N.	4.00	3.30	4.30	4.30	4.20	3.80	4.30	5.10
Uric acid N in gm.		0.0655	0.0624		0.0606		0.0633	
Per cent. of total urine N.		1.07	1.09		1.16		1.25	
Purin N in gm.			0.0248		0.0192		0.0225	
Per cent. of total urine N.			0.43		0.37		0.45	
Albumin gm. per 1,000 c.c.	0.5	0.6	0.3	0.7	0.5	0.8	0.8	0.7
Body weight in kilos.	90.49	90.83	90.72	90.49	90.94	90.94	90.94	91.17

Date.	November, 1910.			December, 1910							
	28	29	30	1	2	3	4	5	6	7	
ogen in gm.	6.05										
er in c.c.											
ries.	2,051.00										
onal N intake						Urea 2 gm. N	Urea 2 gm. N	Urea 2 gm. N			
e in c.c.	1,105.00	1,530.00	1,785.00	1,650.00	1,275.00	1,300.00	1,560.00	1,790.00	1,515.00	1,260.00	83
e specific gravity	1.014	1.014	1.011	1.013	1.014	1.011	1.013	1.012	1.015	1.016	
e N in gm.	5.24	6.70	6.91	6.69	5.93	6.07	7.05	7.01	6.13	5.67	
s N in gm.	0.65	0.73	0.76	1.50	0.42	0.38	0.84	0.97	——	0.19	
l N in gm.	5.89	7.43	7.67	8.19	6.35	6.45	7.89	7.98	——	5.86	
N in gm.	4.38	5.60	5.89	5.50	4.99	5.31	5.98	6.06	5.74	4.37	
nt. of total urine N	83.50	83.50	85.20	82.20	84.10	87.50	85.10	86.40	93.60	77.10	
cid N in gm.											
nt. of total urine N											
N in gm.											
nt. of total urine N.											
in gm. per 1,000 c.c.	0.7	0.7	0.9	0.8	0.5						
veight in kilos.								89.5	89.1	89.1	

								December, 1909.			
15	16	17	18	19	20	21	22	8	9	10	11
5.62	5.72	5.66	5.54	5.66	5.64	5.76	5.72	10.04	10.06	10.18	10.12
1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00
2,410.00	2,418.00	2,414.00	2,404.00	2,414.00	2,412.00	2,422.00	2,418.00	2,408.00	2,409.00	2,419.00	2,414.00
.....	.....	Diuretin ..	.....	Urea 2 gm. N	.....	.....	.....	.....	.....	.....	.....
1,600.00	1,690.00	1,640.00	2,030.00	1,740.00	1,610.00	1,530.00	1,380.00	1,830.00	1,645.00	1,730.00	1,650.00
1.009	1.008	1.009	1.008	1.008	1.009	1.009	1.009	1.013	1.013	1.013	1.004
5.06	5.16	5.20	5.51	5.37	5.21	5.13	5.12	8.76	8.55	9.20	8.80
1.34	1.37	1.49	2.59	1.06	2.30	1.54	0.66	1.54	1.34	1.08	1.27
6.40	6.53	6.69	8.10	6.43	7.51	6.67	5.78	10.30	9.89	10.28	10.07
4.08	4.08	3.77	4.30	4.32	4.26	4.16	3.79	7.55	7.15	7.63	7.52
80.60	79.70	72.50	78.00	80.60	81.80	81.10	74.00	83.90	83.60	82.80	85.40
0.22	0.26	0.21	0.24	0.29	0.26	0.23	0.23	0.26	0.34	0.37	0.39
4.30	5.10	4.00	4.30	5.40	4.90	4.50	4.50	2.90	3.90	4.02	4.40
0.0633	.....	0.0564	.....	0.0598	0.0672	.....	0.0620	0.0715	0.0681	0.0820	.....
1.25	.....	1.09	.....	1.11	1.29	.....	1.22	0.82	0.79	0.90	.....
0.0225	.....	0.0300	.....	0.0324	0.0424	.....	0.0415	0.053	0.073	0.058	.....
0.45	.....	0.58	.....	0.60	0.89	.....	0.87	0.61	0.85	0.64	.....
0.8	0.7	0.7	0.7	0.7	0.7	0.8	0.6	0.7	0.8	0.6	0.7
90.94	91.17	91.40	91.62	91.40	91.17	91.40	91.28	92.64	92.62	92.30	92.08

												January,
6	7	8	9	10	11	12	13	14	15	16	17	23
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	13.00
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,155.00
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,150.00
.....	.....	.....	.....	.....	.....	.....	Urea 2 gm. N	Urea 2 gm. N	Urea 2 gm. N	.....	.....	Urea 2 gm. N
,515.00	1,260.00	830.00	1,125.00	1,184.00	1,240.00	1,130.00	1,290.00	1,565.00	1,900.00	1,595.00	1,708.00	2,440.00
1.015	1.016	1.018	1.015	1.014	1.017	1.019	1.015	1.013	1.012	1.015	1.012	1.013
6.13	5.67	4.20	5.13	5.14	5.28	4.79	5.40	6.36	6.70	5.55	5.49	11.88
——	0.19	0.45	1.14	0.71	1.04	1.04	1.04	0.69	1.69	0.35	0.75	1.31
——	5.86	4.65	6.27	5.85	6.32	5.83	6.44	7.05	8.39	5.90	6.24	13.19
5.74	4.37	3.48	lost	4.30	4.45	4.02	4.51	5.29	lost	4.59	4.39	.....
93.60	77.10	82.90		83.60	84.30	83.90	83.50	83.20		82.70	79.90	.....
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	0.067
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	0.56
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
.....	.....	.....	.....	1.4	1.2	1.4	1.2	0.8	1.3	0.8	0.5	1.0
89.1	89.1	89.0	89.2	89.8	89.9	89.9	90.0	90.35	90.8	90.9	89.7	91.5

12	13	14	15	16	17	18	19	20	21	22
10.12	10.00	10.02	10.00	10.08	10.12	10.14	10.36	10.12	9.92	10.14
1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00
2,414.00	2,405.00	2,406.00	2,405.00	2,411.00	2,414.00	2,416.00	2,433.00	2,414.00	2,398.00	2,416.00
.....	.....	Urea 2 gm. N	.....	.....	.....	.....	.....	.....	Urea 2 gm. N	.....
1,500.00	1,455.00	1,585.00	1,570.00	1,590.00	1,570.00	1,580.00	1,440.00	1,360.00	1,355.00	1,440.00
1.014	1.015	1.014	1.015	1.015	1.015	1.014	1.065	1.016	1.015	1.015
8.95	8.70	9.97	9.62	9.63	9.50	9.43	9.12	8.88	9.18	9.41
1.87	1.58	1.03	1.39	1.75	1.59	0.90	1.99	1.62	1.24	1.17
10.82	10.28	11.00	11.01	11.38	11.09	10.33	10.11	10.50	10.42	10.58
7.78	7.44	8.55	8.44	8.50	8.18	8.12	7.87	7.32	7.85	8.14
86.90	85.80	85.80	87.70	88.20	86.10	86.10	86.30	82.40	85.30	86.30
0.32	0.34	0.35	0.37	0.35	0.35	0.39	0.37	0.37	0.34	0.44
3.60	3.90	3.50	3.80	3.70	3.70	4.10	4.00	3.90	3.70	4.60
0.1327	0.0803	0.0607	0.0890	.....	0.0593	.....	.....	.....	0.0572	.....
1.48	0.93	0.61	0.92	.....	0.74	.....	.....	.....	0.62	.....
.....	0.0648	.....	.....	.....	.....	.....	.....	.....	.....	.....
.....	0.74	.....	.....	.....	.....	.....	.....	.....	.....	.....
0.8	0.9	1.0	0.6	1.1	1.0	0.9	1.0	1.0	1.0	1.0
92.19	92.08	92.19	92.30	92.19	92.30	92.42	92.42	92.30	92.42	92.64

911. February, 1911.

24	25	26	27	28	29	30	31	1	2	3
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Urea gm. N	Urea 2 gm. N	.....	.....	.....	.....	Hypoxanthine 2 gm. N	Hypoxanthine 2 gm. N	Hypoxanthine 2 gm. N	.....	.....
,630.00	2,155.00	2,020.00	1,790.00	1,890.00	1,885.00	2,380.00	2,540.00	2,250.00	1,810.00	2,160.00
1.011	1.012	1.013	1.013	1.013	1.014	1.012	1.010	1.013	1.013	1.013
12.91	11.33	11.95	10.94	11.17	10.95	11.52	11.77	11.73	10.08	11.55
1.28	1.66	2.12	1.43	——	0.69	1.65	1.10	1.83	2.63	1.63
14.19	12.99	14.07	12.37	——	11.64	13.17	12.87	13.56	12.71	13.18
.....	9.31	10.50	9.45	9.52	.....	9.52	10.16	.....	8.31	.....
.....	82.00	87.80	86.40	85.30	.....	82.50	86.40	.....	82.30	.....
0.099	0.051	0.083	0.132	0.133	0.092	0.245	0.384	0.455	0.221	0.292
0.76	0.45	0.69	1.2	1.19	0.84	2.13	3.26	3.88	2.19	2.52
0.013	0.015	0.0126	0.0134	0.0142	0.0118	0.0119	0.0238	0.0240	0.0199	0.0323
0.10	0.13	0.105	0.12	0.13	0.11	0.10	0.20	0.20	0.20	0.28
0.8	1.0	0.8	0.7	1.0	1.0	0.8	1.0	0.9	0.9	1.0
90.5	90.	89.9	89.9	90.1	90.3	90.3	90.3	90.2	90.1	90.6

TABLE III. *Second Series.*

	January, 1910.											
	20	21	22	23	24	14	15	16	17	18	19	20
	10.12	9.92	10.14	10.00	10.00	13.11	12.85	13.07	13.00	12.96	13.00	13.04
	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00
	2,414.00	2,398.00	2,416.00	2,405.00	2,405.00	2,478.00	2,457.00	2,473.00	2,150.00	2,147.00	2,150.00	2,153.00
	.....	Urea 2 gm. N	.....	.....	.....	.....	.....	.....	.....	.....	Urea 2 gm. N	Urea 2 gm. N
	1,360.00	1,355.00	1,440.00	1,420.00	1,390.00	1,475.00	1,680.00	1,655.00	1,790.00	1,630.00	1,975.00	1,780.00
95	1.016	1.015	1.015	1.013	1.014	1.014	1.014	1.015	1.015	1.015	1.014	1.013
	8.88	9.18	9.41	8.93	8.95	11.53	12.43	12.05	12.90	12.10	13.61	13.60
	1.62	1.24	1.17	1.99	1.52	.....	1.71	1.13	2.07	1.30	1.42	1.27
	10.50	10.42	10.58	10.92	10.47	.....	14.14	13.18	14.97	13.40	15.03	14.87
	7.32	7.85	8.14	.....	.....	10.10	10.35	10.22	10.74	10.30	11.36	11.74
	82.40	85.30	86.30	.....	.....	87.60	83.30	83.30	84.00	85.10	83.60	86.40
	0.37	0.34	0.44	.....	.....	0.59	0.36	0.56	0.61	0.30	0.63	0.57
	3.90	3.70	4.00	.....	.....	5.10	2.80	4.70	4.70	2.50	4.10	4.10
	.....	0.0572	.....	.....	.....	.....	0.0977	.....	.....	0.0580	0.0877	0.0807
	.....	0.62	.....	.....	.....	.....	0.78	.....	.....	0.49	0.65	0.60
	.....	.....	.....	.....	.....	.....	0.0491	.....	.....	0.0735	0.0634	0.0616
	.....	.....	.....	.....	.....	.....	0.40	.....	.....	0.61	0.46	0.45
	1.0	1.0	1.0	1.0	1.0	1.1	0.8	0.6	0.9	0.7	1.2	0.9
2	92.30	92.42	92.64	92.76	92.76	92.19	92.30	92.19	92.30	92.42	92.42	92.30

TABLE IV. *Third Series.*

	February, 1911.											
	1	2	3	4	5	6	7	8	9	10	11	12
	.....	.....	.....	.....	.....	.....	.....	.....	.....	13.00	.....	.....
	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,155.00	.....	.....
	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,150.00	.....	.....
	Hypoxanthine 2 gm. N	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	2,250.00	1,810.00	2,160.00	1,870.00	1,860.00	2,020.00	2,220.00	2,140.00	1,975.00	2,160.00	2,335.00	2,070.00
	1.013	1.013	1.013	1.015	1.010	1.014	1.014	1.012	1.013	1.013	1.011	1.013
	11.73	10.08	11.55	10.02	10.39	10.94	10.85	10.22	10.76	10.94	11.22	10.72
	1.83	2.63	1.63	1.57	0.86	1.48	——	1.69	0.74	1.38	2.88	1.42
	13.56	12.71	13.18	11.59	11.25	12.42	——	11.91	11.50	12.32	14.10	12.14
	.....	8.31	.....	8.08	8.63	8.73	.....	9.12	.....	.....	9.09	.....
	.....	82.30	.....	80.60	83.10	79.80	.....	86.00	.....	.....	82.90	.....
	0.455	0.221	0.292	0.136	0.128	0.112	.....	0.104	.....	.....	0.109	.....
	3.88	2.19	2.52	1.35	1.23	1.03	.....	0.99	.....	.....	1.00	.....
8	0.0240	0.0199	0.0323	0.0187	0.0186	0.0384	.....	0.033	.....	.....	0.034	.....
	0.20	0.20	0.28	0.19	0.18	0.35	.....	0.31	.....	.....	0.31	.....
	0.9	0.9	1.0	0.9	1.0	1.0	0.9	0.8	1.3	0.8	0.5	0.5
	90.2	90.1	90.6	90.1	90.3	90.8	91.1	91.0	90.8	91.1	90.9	90.6

21	22	23	24	25	26	27	28	29	30
12.96	13.00	12.70	12.86	13.12	12.92	12.88	12.86	13.14	13.16
800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00
147.00	2,150.00	2,126.00	2,141.00	2,159.00	2,143.00	2,140.00	2,137.00	2,161.00	2,163.00
Urea 2 gm. N	Urea 2 gm. N	.....	.....	.....	.....	.....	.....	.....	.....
875.00	1,790.00	1,765.00	1,815.00	2,355.00	2,120.00	1,780.00	1,665.00	1,960.00	1,940.00
1.014	1.014	1.014	1.013	1.012	1.012	1.014	1.014	1.013	1.013
13.33	13.50	13.26	12.76	13.44	13.04	12.30	11.81	12.56	12.74
1.92	1.04	1.52	0.92	1.22	1.54	1.20	1.54	1.48	0.93
15.25	14.54	14.78	13.68	14.66	14.58	13.50	13.35	14.04	13.67
11.70	11.60	11.42	10.35	10.84	10.52	9.82	9.99	10.39	10.40
87.70	86.00	86.10	81.20	81.10	80.60	79.80	84.60	82.80	81.60
0.55	0.60	0.54	0.70	0.72	0.61	0.54	0.55	0.65	0.53
4.10	4.40	4.10	5.50	5.30	4.60	4.40	4.80	5.20	4.20
0.0879	0.0537	0.1020	.....	0.0757	0.1067	0.0820	.....	.....	0.0880
0.67	0.40	0.77	.....	0.56	0.82	0.67	.....	.....	0.69
0.0658	0.0576	.....	.....	0.0618	.....	.....	.....	.....	0.0402
0.49	0.43	.....	.....	0.46	.....	.....	.....	.....	0.32
0.8	0.9	0.7	0.6	0.8	0.8	1.0	0.9	0.8	0.7
92.42	92.87	92.53	93.21	93.21	92.87	92.76	92.98	93.21	92.98

13	14	15	16	17	18	19	20	21	22	2
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	...
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	...
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	...
Hypoxanthine 2 gm. N	Hypoxanthine 2 gm. N	Hypoxanthine 2 gm. N	.....	.....	.....	.....	.....	.....	.....	...
2,185.00	2,450.00	2,605.00	1,950.00	2,080.00	2,020.00	1,940.00	2,385.00	1,960.00	1,990.00	1,91
1.013	1.013	1.013	1.014	1.013	1.013	1.014	1.013	1.014	1.014	
10.72	11.34	12.20	10.78	11.45	11.02	10.82	11.69	10.94	11.07	1
3.10	0.76	1.41	1.69	——	1.04	1.44	1.25	1.26	1.40	
13.82	12.10	13.61	12.47	——	12.06	12.26	12.94	12.20	12.47	1
8.56	9.42	10.21	.....	.....	9.05	9.16	9.92	9.22	9.39	
80.00	83.00	83.70	.....	.....	82.10	84.60	84.80	84.20	84.80	8
.....	0.458	.....	0.248		0.152	0.119	.....	0.128		...
.....	4.00	.....	2.23		1.38	1.10	.....	1.15		...
.....	0.029	.....	0.029		0.031	0.043	.....	0.0330		...
.....	0.25	.....	0.26		0.28	0.40	.....	0.30		...
0.5	0.4	0.6	0.8	0.6	0.8	0.7	0.7	0.7	0.8	
90.7	90.6	90.7	90.3	90.3	90.2	90.2	90.7	90.3	90.6	9

				February, 1910.							
28	29	30	31	1	2	3	4	5	6	15	16
12.86	13.14	13.16	12.84	13.10	13.22	13.20	13.06	12.86	13.20	13.24	13.
1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	2,190.
2,137.00	2,161.00	2,163.00	2,137.00	2,158.00	2,167.00	2,166.00	2,155.00	2,137.00	2,166.00	2,169.00	2,211.
.....	.....	.....	Nucleic acid 2 gm. N	Nucleic acid 2 gm. N	Nucleic acid 2 gm. N	Nucleic acid 2 gm. N	.....	.....	.....	Uric acid 2 gm. N	Uric 2 gm
1,665.00	1,960.00	1,940.00	1,940.00	1,980.00	1,780.00	1,920.00	2,000.00	1,865.00	1,820.00	1,720.00	1,860.
1.014	1.013	1.013	1.013	1.014	1.014	1.014	1.014	1.013	1.015	1.014	1.
11.81	12.56	12.74	12.98	13.27	13.23	14.05	14.30	13.21	13.15	12.22	13.
1.54	1.48	0.93	1.40	1.45	1.48	1.35	0.98	1.51	1.34	1.40	1.
13.35	14.04	13.67	14.38	14.72	14.71	15.40	15.28	14.72	14.49	13.62	15.
9.99	10.39	10.40	10.72	10.97	10.97	11.48	11.45	10.76	10.61	10.27	10.
84.60	82.80	81.60	82.70	82.70	83.00	81.70	80.00	81.40	80.07	84.10	78.
0.55	0.65	0.53	0.65	0.75	0.75	0.78	0.59	0.78	0.80	0.70	0.
4.80	5.20	4.20	5.00	5.70	5.60	5.60	4.10	5.90	6.10	5.70	5.
o	.....	0.0880	0.1120	0.1453	0.1276	0.1620	0.1860	0.149	0.1638	0.1024	0.
.....	.....	0.69	0.85	1.09	0.91	1.16	1.31	1.13	1.24	0.84	0.
.....	.....	0.0402	0.0606	0.0492	0.0558	0.0703	0.0655	0.052	0.0581	0.0609	.....
.....	.....	0.32	0.47	0.37	0.42	0.50	0.46	0.39	0.44	0.50	.....
0.9	0.8	0.7	1.3	0.5	0.5	0.7	0.7	1.8	1.3	0.8	0.
92.98	93.21	92.98	92.64	92.30	92.42	92.53	92.30	92.87	92.39	92.76	93.

									March, 1911.			
20	21	22	23	24	25	26	27	28	1	2	3	4
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
.....	.....	.....	.....	Inosine 5 gm. N	Inosine 5 gm. N	Inosine 5 gm. N	.....	.....	.....	.....	.....	.....
385.00	1,960.00	1,990.00	1,910.00	2,390.00	2,095.00	2,200.00	1,910.00	2,000.00	1,980.00	2,020.00	2,000.00	2,090.00
1.013	1.014	1.014	1.013	1.012	1.014	1.014	1.013	1.013	1.012	1.013	1.013	1.01
11.69	10.94	11.07	10.56	11.45	11.20	11.85	10.41	11.02	10.58	10.73	10.58	10.24
1.25	1.26	1.40	0.93	1.37	1.08	1.76	1.26	1.32	1.64	——	1.08	2.31
12.94	12.20	12.47	11.49	12.82	12.28	13.61	11.67	12.34	12.22	——	11.66	12.55
9.92	9.22	9.39	8.57	9.37	.....	10.34	8.99	9.10	8.69	.....	8.78	
84.80	84.20	84.80	81.10	81.90	.....	87.30	86.30	82.50	82.10	.....	82.90	
.....	0.128	.....		0.135	0.191	0.195	0.171	0.151	0.128	.....	0.110	
.....	1.15	.....		1.18	1.71	1.64	1.64	1.36	1.2	.....	1.03	
.....	0.0330	.....		0.036	0.013	0.030	0.027	0.019	0.020	.....	.....	.....
.....	0.30	.....		0.31	0.12	0.25	0.26	0.18	0.18	.....	.....	.....
0.7	0.7	0.8	0.7	0.8	0.6	0.5	0.8	0.6	0.7	0.6	0.6	0.9
90.7	90.3	90.6	90.6	90.8	90.6	90.6	90.3	90.2	90.3	90.3	90.3	90.6

	17	18	19	20	21	22	23	7
3	13.12	13.10	13.20	13.32	13.42	13.12	13.20	13.18
0	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00
0	2,159.00	2,158.00	2,166.00	2,175.00	2,183.00	2,159.00	2,166.00	2,164.00
cid N	Uric acid 2 gm. N	Uric acid 2 gm. N	.....	.....	.....	.....	.....	Uric acid 2 gm. N
0	1,875.00	1,900.00	1,990.00	1,955.00	1,980.00	2,205.00	2,230.00	1,900.00
12	1.01	1.01	1.01	1.01	1.012	1.012	1.012	1.014
2	13.82	13.37	13.43	13.21	12.99	12.75	12.91	12.89
5	1.67	1.37	1.55	1.83	0.97	0.99	1.40	1.57
7	15.49	14.74	14.98	15.04	13.96	13.74	14.31	14.46
2	11.40	11.06	10.76	10.85	10.78	10.60	10.58	10.72
0	82.50	82.70	80.00	82.00	82.00	83.10	81.50	83.20
1	0.65	0.64	0.65	0.50	0.59	0.48	0.62	0.51
0	4.70	4.70	4.90	3.80	4.20	3.80	4.80	3.90
187	0.1666	0.1813	0.2127	0.1241	0.1234	0.0903	0.0977	0.1177
8	1.21	1.36	1.58	0.94	0.95	0.71	0.76	0.91
....	0.0632	0.0498	0.0428	0.0570	0.0440	0.0580	0.0640	0.0568
....	0.46	0.37	0.32	0.44	0.34	0.46	0.60	0.44
	0.9	0.8	0.9	0.6	0.5	0.4	0.6	0.9
0	93.44	93.44	93.32	93.44	93.44	93.66	93.32	93.21

	5	6	7	8	9	10	11	12	13	
	.....	.....	.....	.....	.....	.....	.....	.....	.....	
	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,1
	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,1
	.....	Uric acid 6 gm. N	Uric acid 6 gm. N	Uric acid 6 gm. N	.....	.....	.....	.....	.....	...
	1,900.00	2,450.00	2,450.00	2,220.00	2,130.00	2,150.00	1,935.00	1,940.00	1,900.00	2,4
	1.013	1.011	1.012	1.013	1.013	1.013	1.014	1.014	1.015	
	9.74	10.98	11.48	11.92	11.43	11.37	10.53	10.76	10.76	
	0.94	1.64	1.12	1.19	1.24	1.64	1.62	1.16	1.44	
	10.68	12.62	12.60	13.11	12.67	13.01	12.15	11.92	12.20	
	8.30	.....	.....	10.42	9.80	.....	9.13	.....		
	3.80	.....	.....	87.40	85 70	.....	86.70	.....		
	.....	0.109	0.128	0.129	0.121	0.126	0.102	0.114		
	.....	1.0	1.11	1.08	1.06	1.10	0.97	1.04		
	.....	0.021	0.015	0.021	0.024	0.031	0.029	0.019		
	.....	0.19	0.13	0.18	0.20	0.27	0.27	0.18		
	0.8	0.6	0.7	0.7	0.6	0.6	0.6	0.7	0.7	
	90.1	90.2	90.1	89.7	89.8	89.9	89.7	89.4	89.4	

									March, 1910.		
15	16	17	18	19	20	21	22	23	7	8	
13.24	13.28	13.12	13.10	13.20	13.32	13.42	13.12	13.20	13.18	13.28	1
1,800.00	2,190.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,800.00	1,80
2,169.00	2,211.00	2,159.00	2,158.00	2,166.00	2,175.00	2,183.00	2,159.00	2,166.00	2,164.00	2,172.00	2,14
Uric acid 2 gm. N	Uric acid 2 gm. N	Uric acid 2 gm. N	Uric acid 2 gm. N	.....	.....	.....	.....	.....	Uric acid 2 gm. N	Uric acid 2 gm. N	Uri 2 g
1,720.00	1,860.00	1,875.00	1,900.00	1,990.00	1,955.00	1,980.00	2,205.00	2,230.00	1,900.00	2,060.00	1,87
1.014	1.012	1.01	1.01	1.01	1.01	1.012	1.012	1.012	1.014	1.012	
12.22	13.12	13.82	13.37	13.43	13.21	12.99	12.75	12.91	12.89	13.64	1
1.40	1.95	1.67	1.37	1.55	1.83	0.97	0.99	1.40	1.57	1.61	
13.62	15.07	15.49	14.74	14.98	15.04	13.96	13.74	14.31	14.46	15.25	1
10.27	10.32	11.40	11.06	10.76	10.85	10.78	10.60	10.58	10.72	11.61	1
84.10	78.70	82.50	82.70	80.00	82.00	82.00	83.10	81.50	83.20	85.10	8
0.70	0.71	0.65	0.64	0.65	0.50	0.59	0.48	0.62	0.51	0.54	
5.70	5.40	4.70	4.70	4.90	3.80	4.20	3.80	4.80	3.90	3.90	
0.1024	0.1187	0.1666	0.1813	0.2127	0.1241	0.1234	0.0903	0.0977	0.1177	0.0783	
0.84	0.98	1.21	1.36	1.58	0.94	0.95	0.71	0.76	0.91	0.58	
0.0609	.....	0.0632	0.0498	0.0428	0.0570	0.0440	0.0580	0.0640	0.0568	0.0615	
0.50	.....	0.46	0.37	0.32	0.44	0.34	0.46	0.60	0.44	0.45	
0.8	0.8	0.9	0.8	0.9	0.6	0.5	0.4	0.6	0.9	0.6	
92.76	93.10	93.44	93.44	93.32	93.44	93.44	93.66	93.32	93.21	93.55	9

											April, 1911.	
3	4	5	6	7	8	9	10	11	12	13	3	4
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	13.00	.....
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,155.00	.....
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	2,150.00	.....
.....	.....	.....	Uric acid 6 gm. N	Uric acid 6 gm. N	Uric acid 6 gm. N	.....	.....	.....	.....	.....	.....	.....
000.00	2,090.00	1,900.00	2,450.00	2,450.00	2,220.00	2,130.00	2,150.00	1,935.00	1,940.00	1,900.00	2,420.00	2,250.00
1.013	1.013	1.013	1.011	1.012	1.013	1.013	1.013	1.014	1.014	1.015	1.013	1.01
10.58	10.24	9.74	10.98	11.48	11.92	11.43	11.37	10.53	10.76	10.76	11.95	11.53
1.08	2.31	0.94	1.64	1.12	1.19	1.24	1.64	1.62	1.16	1.44	1.78	1.04
11.66	12.55	10.68	12.62	12.60	13.11	12.67	13.01	12.15	11.92	12.20	13.73	12.57
8.78	8.30		.....	.....	10.42	9.80	.....	9.13	.....		9.97	.....
82.90	83.80		.....	.....	87.40	85.70	.....	86.70	.....		83.40	.....
0.110		.....	0.109	0.128	0.129	0.121	0.126	0.102	0.114		0.132	0.1
1.03		.....	1.0	1.11	1.08	1.06	1.10	0.97	1.04		1.10	1.2
.....	.....	.....	0.021	0.015	0.021	0.024	0.031	0.029	0.019		0.076	0.0
.....	.....	.....	0.19	0.13	0.18	0.20	0.27	0.27	0.18		0.63	0.5
0.6	0.9	0.8	0.6	0.7	0.7	0.6	0.6	0.6	0.7	0.7	0.8	0.5
90.3	90.6	90.1	90.2	90.1	89.7	89.8	89.9	89.7	89.4	89.4	90.1	90.0

9	10	11	12
.98	12.88	13.14	13.08
.00	1,800.00	1,800.00	1,800.00
.00	2,140.00	2,164.00	2,156.00
acid n. N	Uric acid 2 gm. N	.....	.....
.00	1,810.00	1,740.00	1,670.00
.014	1.014	1.015	1.014
.50	13.46	13.46	12.20
.46	1.87	1.21	1.49
.96	15.33	14.67	13.69
.32	11.15	11.12	10.15
.90	82.80	82.60	83.20
.53	0.45	0.52	0.41
.90	3.40	3.80	3.40
.0850	0.1030	0.1853	0.0861
.63	0.76	1.37	0.71
.0605	.....	.....	.....
.45	.....	.....	.....
.6	0.8	0.7	1.1
.21	93.10	92.76	92.64

	5	6	7	8	9
	.....	.....	.....	.....	.....
	.....	.....	.....	.....	.....
	.....	.....	.....	.....	.....
	.....	.....	Guanosine 6.2 gm. N	Guanosine 6.2 gm. N	.....
	2,410.00	2,400.00	2,170.00	2,380.00	2,315.00
	1.013	1.013	1.015	1.013	1.014
	11.62	12.76	12.42	13.50	12.32
	1.83	1.49	2.46	0.82	1.46
	13.45	14.25	14.88	14.32	13.78
	9.60	10.85	10.52	11.66	10.34
	83.40	85.00	84.50	86.40	84.00
	0.132	0.177	0.154	0.202	0.165
	1.14	1.38	1.24	1.50	1.33
	0.077	0.017	0.018	0.086	0.077
	0.60	0.13	0.15	0.64	0.62
	0.5				
	89.9	89.7	89.4	89.2	89.4

THE DURATION OF IMMUNE BODIES IN THE BLOOD AFTER ANTITYPHOID INOCULATION.*

By MARTHA WOLLSTEIN, M.D.

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Inoculation with dead typhoid bacilli for the prevention of typhoid fever has been successfully practised in the armies of England, Germany, the United States, and Japan. The literature on the subject has recently been reviewed by Major Russell[1] and need, therefore, not be repeated here.

Russell concludes that the incidence of typhoid fever is six to fifteen times as high among unvaccinated soldiers as among those who have been inoculated with the antityphoid vaccine. This clinical immunity to the disease is expressed in the reactions of the serum of inoculated persons to bactericidal, opsonic, and agglutination tests with the typhoid bacillus, the immune bodies appearing in the blood from the fifth to the ninth day after inoculation.

The general impression that immunity after typhoid vaccination lasts about two years seems to be based upon Wright's[2] original statement that "it is not infrequent to find an agglutinating power in the blood of inoculated persons as long as two years after the inoculation of antityphoid vaccine." Harrison[3] found evidence of bactericidal activity higher than normal and of agglutinating power in the serum of one man who had been inoculated four years previously, and in another six years after vaccination. But the reactions were very low, agglutinins in dilutions of 1 to 30 in the former case and 1 to 20 in the latter, while bactericidins were found in dilutions of 1 to 60 and 1 to 40, respectively. Moreover, these two

* Received for publication, May 29, 1912.

[1] Russell, F. F., *Boston Med. and Surg. Jour.*, 1911, clxiv, 1.

[2] Wright, A. E., A Short Treatise on Antityphoid Inoculation, Westminster, 1904, 64.

[3] Harrison, W. S., *Jour. Roy. Army Med. Corps*, 1907, viii, 472.

cases had not been followed systematically and their serum reactions to the typhoid bacillus before inoculation are unknown.

Russell[4] followed "a large number" of cases through weekly blood tests, and found that while agglutinins were sometimes increased on the fourth day, they were always marked by the sixth or eighth day, shooting up rapidly to ten or even twenty thousand. The fall began in about six weeks, and in one case after fifteen months agglutination was present in dilutions of 1 to 80 or 1 to 100. Russell[5] also found that the phagocytic power of the serum might exceed the normal after a year.

Because of this vagueness in the literature on the duration of immunity after antityphoid inoculation, it seemed worth while to make a systematic study of a definite number of individuals for as prolonged a period of time as seemed necessary. Through the courtesy of Captain H. J. Nichols, of the Army Medical Corps, it was possible to examine the blood of twenty-two soldiers stationed in and around New York City. In addition, the serum of two physicians was studied, making in all twenty-four persons who had received antityphoid inoculations.

The tests were made before vaccination, ten days after each inoculation, and then at intervals over a period of thirteen months. Dr. Nichols very kindly made the inoculations and obtained the blood specimens. Three injections were given to each man, the first of approximately five hundred million dead bacilli, the second and third of one thousand million, at ten day intervals. The vaccine was obtained by Dr. Nichols from the Army Medical School in Washington, and was prepared from a non-virulent strain of typhoid bacillus killed by heating at 60° C. for one hour. One per cent. tricresol was added to the vaccine.

ANIMAL EXPERIMENTS.

Just before this study was begun, Bassenge[6] had published his observations on the ability of lecithin to increase the immunizing power of the typhoid bacillus, and Wassermann and Seitz[7] had

[4] Russell, F. F., *Bull. Johns Hopkins Hosp.*, 1910, xxi, 83.

[5] Russell, F. F., *Bull. Johns Hopkins Hosp., loc. cit.*

[6] Bassenge, R., *Deutsch. med. Wchnschr.*, 1908, xxxiv, 139.

[7] Wassermann, M., and Seitz, A. M., *Deutsch. med. Wchnschr.*, 1908, xxxiv, 2175.

failed to confirm the statements of Bassenge, their experiments leading them to the conclusion that lecithin causes only a local increase in resistance which is short in duration and absolutely non-specific in character. Our preliminary experiments with white rats were not encouraging, since vaccine plus 1 per cent. lecithin produced a serum that agglutinated typhoid bacilli in dilutions of 1 to 2,500, and was bactericidal in a dilution of 1 to 40,000, while a similar dose without lecithin developed a lytic action that was twice as strong. Again, lecithin in 10 per cent. solution did not kill the typhoid bacillus in forty-eight hours, an observation which was confirmed by Vallet and Rimbaud,[8] who found the bacilli viable after three days' contact with 10 per cent. lecithin. Nor did they find that the addition of a lecithin emulsion to typhoid bacilli produced a serum whose agglutinating power was superior to that obtained from the bacilli alone. It was possible, however, that lecithin might lessen the local reaction, always more or less painful after an antityphoid vaccination, and the experiment of combining lecithin and vaccine was tried on a monkey.

Experiment 1.—A rhesus monkey was inoculated subcutaneously into the right thigh with 1 c.c. of vaccine, which equalled one agar slant of typhoid bacilli killed with 0.04 per cent. lysol. The temperature before inoculation was 40.6° C., and was still the same after two hours. The serum of this monkey did not agglutinate bacilli in a dilution of 1:20, and was bactericidal in dilutions of 1:80.

After twenty-four hours the temperature of the animal was 40.2° C. The thigh was held stiffly and there was a small amount of induration about the point of inoculation. There was no redness. The animal was lively. Next day the temperature was the same and the animal used the leg well. There was no tenderness. Thereafter the monkey seemed perfectly well, and the induration disappeared.

Six days after inoculation the blood agglutinated the bacilli in dilutions of 1:40, but gave a positive bactericidal effect in dilutions of 1:5,120. On the eighth day the animal was reinoculated with double the first dose of vaccine, after which the temperature did not rise, and no pain, tenderness, or swelling was apparent in the thigh. Six days later agglutinins were present in dilutions of 1:640, and bactericidins in dilutions of 1:5,120.

Experiment 2.—A rhesus monkey was inoculated subcutaneously into the right thigh with 1 c.c. of vaccine plus 1 c.c. of 1 per cent. lecithin. The temperature before inoculation was 40.6° C. The serum did not agglutinate the typhoid bacilli in a dilution of 1:20, but killed them in dilutions of 1:80. The fol-

[8] Vallet, G., and Rimbaud, L., *Compt. rend. Soc. de biol.*, 1910, lxviii, 302.

lowing day the temperature had not risen; the thigh felt warm and indurated, but it was not tender. The monkey held it stiffly for two days. The temperature rose to 41° C. on the second day; then fell to 40.5° C. on the third day. The induration was gone on the third day. After six days agglutinins were present in dilutions of 1:20, and bactericidins in dilutions of 1:40,960. After a second dose, double the first in size, the monkey remained perfectly well, and no redness, pain, or swelling appeared. After six days the blood agglutinated the typhoid bacilli in dilutions of 1:2,560, and killed the organisms in dilutions of 1:367,680.

The addition of lecithin to the vaccine did not influence the local reaction in this monkey, but it must be admitted that in both experiments the local signs were very slightly marked. Immune bodies were present in higher dilutions in the serum of the animal to which lecithin had been given. On repeating these two experiments, the local reactions were practically similar to those in the first set, but the immune bodies were slightly less marked in the case of the monkey that received lecithin with the vaccine. The question of individual variation of monkeys to typhoid infection evidently enters largely into these results, and lecithin would seem to be inconstant in its action, if it has any at all, as a promoter of typhoid antibody formation.

Since the subcutaneous injection of a 1 per cent. lecithin emulsion, either alone or with antityphoid vaccine, had had no bad effects in rats or monkeys, and since there was still a slight possibility that lecithin added to the vaccine might help to increase the production of agglutinins and bactericidins, it was decided to divide the twenty-four human cases into two groups of twelve; one group was to receive antityphoid vaccine alone, while the other was to receive vaccine plus a 1 per cent. lecithin emulsion. It may be said at once that the lecithin did not, in any case, lessen the pain or the duration of the local reaction, nor, on the other hand, did it in a single instance increase the local signs.

SERUM REACTIONS IN THE INOCULATED PERSONS.

The immunity to typhoid fever depends apparently upon bacteriolytic antibodies in the blood; hence it follows that tests for bacteriolysins or bactericidins are of the greatest importance in judging whether or not a given serum is immune to the typhoid bacillus.

Whether or not the presence of agglutinins adds to the protective power of such a serum we do not know, but it is certain that bactericidins and agglutinins in antityphoid sera do not always run in parallel lines.

In studying the sera from the twenty-four vaccinated persons, bactericidal tests were made according to the method of Stern and Korte,[9] and agglutinins were also looked for. Phagocytic tests were not made. Russell[10] believes that bactericidal tests are valueless because of the variability of the same strain of the typhoid bacillus to bactericidal action. The strain that we used throughout this work was one that had given good results with preliminary tests on antityphoid goat and rabbit sera. In order to eliminate errors as far as possible, it was our custom to make a test with goat or rabbit serum before examining each set of the human specimens. Schlemmer[11] has recently found that some strains of typhoid bacillus are very resistant to the action of bactericidal sera, others less so, but that the degree of serum resistance remains constant for a single strain.

Before the inoculations were begun, preliminary blood tests were made in eighteen cases. Sixteen were negative both as regards agglutinins and bactericidins. One soldier gave a negative agglutination test but a positive bactericidal reaction in a dilution of 1 to 20 only. The serum of another man agglutinated the typhoid bacillus in a dilution of 1 to 40, and killed the organism even when diluted three hundred times. The latter soldier had had typhoid fever three years before. Three other men had previously had a typhoid attack, but their sera were quite devoid of antibodies.

After the first inoculation the lecithin vaccines seemed to give results which were slightly inferior to those produced by the simple suspensions of dead bacilli, four cases giving negative tests with lecithin as against three cases without lecithin. The highest bactericidal titer was one in ten thousand. Only four persons to whom lecithin was given reacted in a dilution of 1 to 300 or over, while of those to whom no lecithin had been given five reacted in dilutions of 1 to 600 and more.

[9] Stern, R., and Korte, W., *Berl. klin. Wchnschr.*, 1904, xli, 213.
[10] Russell, F. F., *Bull. Johns Hopkins Hosp., loc. cit.*
[11] Schlemmer, *Ztschr. f. Immunitatsforsch., Orig.*, 1911, ix, 149.

Ten days after the second dose had been administered there were still three persons with sera having no bactericidins, and each of these persons had received the lecithinated vaccine. Two of these sera, however, contained agglutinins in dilution of 1 to 160 and 1 to 640, respectively. Seven of the sera of persons injected with vaccines containing lecithin killed typhoid bacilli in dilutions of 1 to 5,000 and over, while ten sera of persons vaccinated without lecithin reached as high a titer.

The third inoculation with lecithin raised one of the negative cases to a bactericidal titer of 1 to 40,000, while the other two still remained negative. One serum was bactericidal when diluted a million times, the highest point reached by any specimen. It was obtained from a physician who had had typhoid fever eleven years before the vaccination.

One month after the third inoculation, which was two months after the vaccinations were begun, the immune bodies were present in the sera in the highest amount and thereafter steadily decreased, so that four months after the first inoculation only one specimen reached the 5,000 mark, and that came from a man to whom lecithin vaccine had been given.

After eight months one serum still showed a bactericidal titer of 1 to 2,500, and it came from a soldier to whom lecithin had been administered.

After ten months only fourteen specimens were available for study, as some of the men had been sent elsewhere for duty. Of the fourteen sera, one from a man to whom no lecithin had been given reached a dilution of 1 to 160, while six other sera, four from men who had received lecithin and two from those who had not, failed to react at all.

After thirteen months fourteen sera were again obtained, one of which was bactericidal in a dilution of 1 to 1,280. Ten were negative, two reacted in a dilution of 1 to 40, and one in a dilution of 1 to 80.

Agglutinins dropped out even earlier than did the bactericidins. After four months only two sera agglutinated the typhoid bacillus in a dilution of 1 to 160, and no agglutinins were present in any one

of the fourteen specimens examined thirteen months after vaccination.

DISCUSSION OF RESULTS OBTAINED.

The average bactericidal curve of the antityphoid immune serum resulting from inoculation with dead typhoid bacilli in this series of cases would seem to have been as follows.

Starting from a negative preliminary reaction, the bactericidins appeared after the first inoculation in dilutions of 1 to 80 to 1 to 2,500, with an exceptional dilution of 1 to 10,000; they rose to 1 to 5,000 or 1 to 20,000 after the second dose, remained there or reached 1 to 40,000 after the third inoculation, and remained at that point or rose, exceptionally, to 1 to 80,000 during the following month. Then, two months after the first inoculation, the highest point was reached and the drop began. This was apt to be abrupt and, as a rule, it went below 1 to 5,000, sometimes even to 1 to 160. After four months a titer of 1 to 2,000 was occasionally present, but the majority of sera fell below 1 to 300 and some (four) had already become negative. Eight months after inoculation the conditions were about the same, but after ten months no bactericidal effect was obtained in any dilution above 1 to 160; the majority lay between 1 to 40 and 1 to 80, and at this time more were negative. At the end of thirteen months the great majority of cases gave an entirely negative bactericidal test (ten out of fourteen); a titer of 1 to 40 or 1 to 80 was sometimes noted, and 1 to 1,280 was the single exception. In this last instance lecithinated vaccine had been administered.

Comparing the results from vaccines with and without lecithin, we find that the examples without lecithin developed bactericidins a little more rapidly, but in no greater quantity than the others, while the examples with lecithin seemed to retain the height slightly better. Two months after the first inoculation ten sera from persons vaccinated with lecithin as against eight vaccinated without it reacted in dilutions above ten thousand. The drop in activity was about as rapid in one case as in the other; e. g., five lecithin cases against four inoculated without lecithin reacted in dilutions greater than 1 to 2,000 at the end of three months. But after four months

three negative cases would seem to show that the sera after lecithin vaccination retained their immune bodies for a shorter period.

At the end of thirteen months nine of the twelve lecithin cases had become negative; one reacted in a dilution of 1 to 80, another in a dilution of 1 to 1,200, and the third could not be tested. It had not been available for study since four months after the beginning of the inoculations, and its low titer of 1 to 160 at that time argued strongly in favor of its being negative later.

The sera obtained from the soldiers inoculated without the addition of lecithin gave no negative bactericidal reactions until ten months after the first inoculations, when two cases failed to kill typhoid bacilli in any dilution, and three months later four other cases were negative. The other two sera examined at this time gave positive results in dilutions of 1 to 40 and 1 to 80 only, and the four cases not available at that time had been dropping steadily since their last examination (tables I and II).

A word remains to be said about the four persons in the series who had suffered attacks of typhoid fever from three to fifteen years before being inoculated. The serum of only one, the most recent case, gave a positive preliminary bactericidal test. This serum rose to a titer of 1 to 10,000 after the first dose of vaccine and reached its highest point, 1 to 40,960, two months later. It then dropped gradually, reaching its original strength in eight months, and after thirteen months was negative. Whether this serum would have fallen to zero in that time if the man had not been vaccinated, is a question. Another man, inoculated eleven years after an attack of typhoid, developed bactericidins which were active in dilutions of 1 to 10,000 after the first vaccination, 1 to 40,000 after the second, and 1 to 1,000,000 after the third. They fell to 1 to 10,000 in another month, remaining at 1 to 5,000 for two months and then gradually falling to 1 to 80, still reacting in that dilution fifteen months after the initial inoculation.

This instance, more than any other of the four under discussion, illustrates the point that when the human organism has once been infected with the typhoid bacillus or its toxin, reinoculation more readily results in the formation of immune bodies to that bacillus.

TABLE I.

Antityphoid Vaccination with Lecithin.

No. of case.	Preliminary test.		10 days after 1st vaccination.		10 days after 2d vaccination.		10 days after 3d vaccination.		2 mos. after 1st vaccination.		3 mos. after 1st vaccination.	
	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.
1	0	0	1:80	0	1:10,240	1:320	1:10,240	1:320	1:40,960	1:640	1:2,560	1:640
2	0	0	1:80	1:80	1:20,480	1:2,560	1:40,960	1:2,560	1:40,960	1:640	1:320	1:320
3[12]	0	0	1:80	0	1:320	1:320	1:640	1:640	1:163,840	1:1,280	1:640	0
4	0	0	0	0	0	Not made	0	0	1:1,280	1:20	1:320	1:20
5	0	0	1:320	Not made	1:10,240	Not made	1:20,480	1:80	1:40,960	1:80	1:1,280	1:80
6	0	0	1:320	Not made	1:10,240	1:320	1:40,960	1:640	1:40,960	1:20	1:80	1:80
7[12]	0	0	1:80	1:320	1:5,120	1:640	1:5,120	1:640	1:40,960	0	1:10,240	Not made
8	0	0	0	1:80	0	1:160	1:40,960	1:320	1:40,960	1:320	1:5,120	Not made
9	0	0	0	1:40	1:20,480	Not made	1:40,960	1:1,280	1:40,960	1:80	1:1,280	Not made
10	0	0	1:10,240	1:40	1:2,560	1:640	1:5,120	1:20	1:40,960	1:640	1:40,960	Not made
11[12]	0	0	1:10,240	1:80	1:40,960	1:320	1:1,210,720	1:80	1:10,240	1:80	1:5,120	1:20
12	1:20	0	0	1:640	0	1:640	0	1:640	1:1,280	1:160	1:640	0

TABLE II.

Antityphoid Vaccination without Lecithin.

No. of case.	Preliminary test.		10 days after 1st vaccination.		10 days after 2d vaccination.		10 days after 3d vaccination.		2 mos. after 1st vaccination.		3 mos. after 1st vaccination.	
	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.
1	0	0	1:160	1:160	1:10,240	Not made	1:10,240	1:320	1:20,480	1:640	1:1,280	1:80
2	0	0	1:80	1:20	1:5,120	1:640	1:5,120	1:640	1:40,960	1:640	1:320	1:160
3	0	0	1:640	1:40	1:20,480	1:640	1:20,480	1:640	1:81,920	1:640	Not made	
4	0	0	1:2,560	1:20	1:20,480	1:320	1:40,960	1:640	1:10,240	1:80	1:20,480	1:160
5	Not made		0	1:20	1:20,480	1:2,560	1:20,480	1:1,280	1:20,480	Not made	1:20,480	1:160
6[13]	1:320	1:40	1:10,240	1:80	1:10,240	1:80	1:10,240	1:80	1:40,960	1:1,280	1:1,280	Not made
7	Not made		0	1:640	1:20,480	Not made	1:20,480	1:320	1:40,960	1:320	Not made	
8	Not made		1:80	1:160	1:40,960	1:320	1:40,960	1:640	1:40,960	1:640	1:5,120	1:320
9	Not made		1:640	1:160	1:5,120	1:320	1:2,560	1:1,280	1:40,960	1:160	1:640	1:160
10	Not made		0	1:640	Not made		1:20,480	1:640	1:80	1:80	1:1,280	1:80
11	Not made		1:2,560	Not made	1:20,480	1:640	1:20,480	1:640	1:160	1:320	1:320	1:640
12	0	0	Not made		Not made		1:20,240	1:640	1:640	1:80	1:40,960	1:80

[12] Nos. 3, 7, and 11 had had typhoid fever.

[13] No. 6 had had typhoid fever.

TABLE I.

oid Vaccination with Lecithin.

mos. after 1st vaccination.		3 mos. after 1st vaccination.		4 mos. after 1st vaccination.		8 mos. after 1st vaccination.		10 mos. af vaccina
Bacteri-cidal.	Agglutination.	Bacteri-cidal.	Agglutination.	Bacteri-cidal.	Agglutination.	Bacteri-cidal.	Agglutination.	Bacteri-cidal.
:40,960	1:640	1:2,560	1:640	1:160	0	1:160	1:80	1:40
:40,960	1:640	1:320	1:320	1:80	Not made	1:160	1:20	0
:163,840	1:1,280	1:640	0	1:80	Not made	Not made		0
:1,280	1:20	1:320	1:20	0	0	Not made		Not m
:40,960	1:80	1:1,280	1:80	1:40	0	Not made		Not m
:40,960	1:20	1:80	1:80	0	Not made	Not made		Not m
:40,960	0	1:10,240	Not made	0	Not made	1:80	0	1:40
:40,960	1:320	1:5,120	Not made	1:1,280	Not made	1:1,280	1:80	Not m
:40,960	1:80	1:1,280	Not made	1:640	1:80	0	0	0
:40,960	1:640	1:40,960	Not made	1:80	1:20	1:320	Not made	0
:10,240	1:80	1:5,120	1:20	1:5,120	0	1:320	0	1:80
:1,280	1:160	1:640	0	1:160	0	Not made		Not m

TABLE II.

d Vaccination without Lecithin.

mos. after 1st vaccination.		3 mos. after 1st vaccination.		4 mos. after 1st vaccination.		8 mos. after 1st vaccination.		10 mos. a vaccina
acteri-cidal.	Agglutination.	Bacteri-cidal.	Agglutination.	Bacteri-cidal.	Agglutination.	Bacteri-cidal.	Agglutination.	Bacteri-cidal.
20,480	1:640	1:1,280	1:80	1:80	1:40	1:80	1:40	Not m
40,960	1:640	1:320	1:160	1:320	1:160	1:160	1:80	1:40
81,920	1:640	Not made		1:2,560	1:80	Not made		Not m
10,240	1:80	1:20,480	1:160	1:1,280	1:40	1:640	1:40	1:80
20,480	Not made	1:20,480	1:160	1:1,280	Not made	1:640	1:80	1:40
40,960	1:1,280	1:1,280	Not made	1:640	1:40	1:320	1:20	1:80
40,960	1:320	Not made		Not made		1:640	0	0
40,960	1:640	1:5,120	1:320	1:2,560	1:80	1:2,560	0	1:160
40,960	1:160	1:640	1:160	1:320	Not made	1:320	0	0
80	1:80	1:1,280	1:80	1:640	1:20	Not made		Not
160	1:320	1:320	1:640	1:320	1:20	Not made		Not
40	1:80	1:40,960	1:80	1:20	1:160	Not made		Not

er 1st on.	13 mos. after 1st vaccination.	
Agglutination.	Bactericidal.	Agglutination.
1:20	0	0
0	0	0
0	Not made.	
ade	Not made.	
ade	0	0
ade	Not made.	
0	0	0
ade	1:1280	0
0	0	0
0	Not made.	
0	1:80	0
ade	Not made.	

ter 1st ion.	13 mos. after 1st vaccination.	
Agglutination.	Bactericidal.	Agglutination.
ade	Not made	
0	0	0
ade	Not made	
0	0	0
1:20	1:40	0
0	0	0
0	0	0
0	1:80	0
0	Not made	
ade	Not made	
ade	Not made	
ade	0	0

Cole[14] proved this point experimentally in animals, and it is the principle upon which the value of typhoid vaccination rests.

It should be remarked that besides these two men, both of whom had suffered from typhoid fever, only one other reacted in a dilution of 1 to 10,000 after the first dose of vaccine. The serum of the third man who had had typhoid reacted slowly. It was active only in a dilution of 1 to 80 after the first dose, but two months later reached a titer of 1 to 160,000, then dropped gradually in ten months to zero. The strength of the serum of the fourth man rose very slowly until it was active in a dilution of 1 to 40,000, and then in four months dropped rapidly to zero. The attack of typhoid fever in this instance had occurred about fifteen years before.

An exceptionally slow and low reaction was obtained in one soldier whose serum failed to show any bactericidins or agglutinins even after the third inoculation of vaccine. One month later bactericidins were present in dilutions of 1 to 1,200 only, and agglutinins in a dilution of 1 to 20. Three months after the initial dose had been given, the reaction was positive in a dilution of 1 to 300, and after four months it had entirely disappeared. The serum of another man failed to respond to the first and second doses, but reached a titer of 1 to 40,000 after the third dose, and proved to be the only one that contained bactericidins in dilutions of 1 to 1,200 thirteen months later.

SUMMARY.

In this series of twenty-four persons inoculated with antityphoid vaccine, the immune bodies in the blood reached their height within two months after the first inoculation, or one month after the third, then fell rapidly within the next two months. Only nineteen of the cases could be followed longer, and eight of these were negative for bactericidins within ten months after inoculation, and fifteen were negative after thirteen months. Only one serum reacted in a dilution of 1 to 1,200 at the end of thirteen months.

The addition of lecithin to the vaccine did not influence the local reaction after inoculation, nor did it appreciably affect the formation of immune bodies to the typhoid bacillus.

[14] Cole, R., *Ztschr. f. Hyg.*, 1904, xlvi, 371.

CONCLUSIONS.

There is, of course, no justification for the conclusion that clinical immunity can be determined absolutely by the measure of immune bodies in the blood, since experience has apparently proved the contrary, a fact which can be accounted for by the latent power of the body cells to react more quickly to a stimulus which has once made them sensitive. That this power lasts for many years after an attack of typhoid fever is well known. Further study is required to prove its duration after antityphoid inoculation. It would seem, however, that reinoculation with typhoid vaccine within a year is indicated when exposure to typhoid fever seems imminent.

POST-HEMORRHAGIC ANEMIA.*

By LINDSAY S. MILNE, M.D.

(*From the Russell Sage Institute of Pathology, New York.*)

PLATES 39-42.

In recent years there has been considerable controversy as to the relation of the anemia that follows hemorrhage to that produced by toxins. A good deal of the confusion has arisen from the attempt to determine, in the case of pernicious anemia, whether the blood picture in this disease is the result of some blood-destroying toxin or whether it is due merely to the loss of blood and the consequent efforts at regeneration.

Anemia Due to Toxins.—The administration of such substances as phenylhydrazin certainly produces appearances almost identical with those in pernicious anemia. In these toxic anemias there is a marked reduction in the number of the red corpuscles. The hemoglobin is greatly reduced, generally in proportion to the number of red cells, but sometimes it is proportionately increased. Polychromatophilia and many nucleated red cells are generally observed. A condition of punctate basophilia in some of the erythrocytes is also of common occurrence. Extramedullary development of myeloid tissue in the spleen and liver is readily produced and resembles closely what may be found in cases of pernicious anemia.

Anemia Following Hemorrhage.—In the anemia that follows hemorrhage certain differences have been described which some authors have considered to be fundamental. Itami (1), Ritz (2), Morawitz (3), and Meyer (4) found that in the toxic anemias there was a much more rapid regeneration of blood than in hemorrhagic anemia of apparently the same severity. In rabbits made anemic by phenylhydrazin or some other toxin, these observers found evidences of myeloid formation in the spleen and liver after eight to ten days, but after hemorrhage there was no extramedullary myeloid metaplasia until three to four weeks had elapsed; even then the changes

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were slight and were often not seen at all. The greater rapidity of regeneration in the toxic group was considered by Itami to be due to the retention in the body of some of the products of disintegration of the red blood corpuscles. Although his results are not above criticism, Itami found in hemorrhagic anemia that the rate of regeneration and the development of extramedullary myeloid metaplasia could be accelerated by the injection of washed red blood corpuscles into the peritoneum of the animal, and that the injection of serum produced no such change. The hemoglobin index in cases of anemia from bleeding is not increased and is generally lower than normal, due to the fact that the regeneration of hemoglobin is much slower than that of the erythrocytes.

Although polychromatophilia is a constant feature of the regeneration of blood after hemorrhage, punctate basophilia of the erythrocytes has apparently been noted only in anemia produced by toxins. Indeed these basophilic granular erythrocytes have come to be regarded as indicative of some toxic process, and are, therefore, considered as affording evidence that hemorrhagic and toxic anemias are not identical.

To determine whether hemorrhage leads to a distinct type of anemia, and to study the regenerative processes in the blood of animals having a hemorrhagic anemia, twenty-four rabbits and six cats were made anemic by repeated bleeding. From some of the animals small amounts of blood were taken daily, from others large amounts daily, and from still others large amounts at longer intervals. The blood was always taken from the ear veins.

The following three tables indicate some of the changes in the blood after hemorrhage, and show to what a marked extent the blood is capable of regeneration.

After bleeding, the hemoglobin takes very much longer than the erythrocytes to return to the normal amount. Two or three days after the beginning of the bleeding, very large numbers of nucleated red cells usually appear in the blood. Later, in the exhaustion stages of the anemia, the number of nucleated erythrocytes is very much less. Anisocytosis is marked, some of the red cells being very small, but many very large. These new cells have a distinctly

basophilic staining reaction and seem to be deficient in hemoglobin. As far as can be judged by staining, these large basophilic cells seem gradually to acquire hemoglobin and eventually differ in no way from normal red blood corpuscles.

TABLE I.

Rabbit 1.

Date.	Amount of blood removed, in c.c.	Per cent. of hemoglobin.	Red blood corpuscles.	Remarks.
Sept. 13, 1911	10	89	5,160,000	Weight, 1,605 gm.
Sept. 14, 1911	10	68	2,870,000	No nucleated red cells.
Sept. 15, 1911	10	75	3,990,000	No nucleated red cells.
Sept. 16, 1911	10	58	3,070,000	Occasional basophilic, stippled red cells, but no nucleated red cells.
Sept. 18, 1911	10	42	2,660,000	
Sept. 19, 1911		37	2,700,000	
Sept. 22, 1911	10	45	3,370,000	
Sept. 25, 1911		43	3,920,000	
Sept. 27, 1911		45	4,180,000	No stippled red cells.
Oct. 2, 1911		65	5,430,000	
Oct. 5, 1911	10	62	4,400,000	
Oct. 7, 1911	10	57	4,060,000	
Oct. 9, 1911	10			Weight, 1,620 gm.
Oct. 11, 1911	10	50	4,200,000	
Oct. 13, 1911	10	48	4,500,000	A few finely stippled red cells. No nucleated red cells.
Oct. 16, 1911	10	58	4,360,000	
Oct. 18, 1911	10	57	4,850,000	Large numbers of stippled red cells.
Oct. 20, 1911	10	55	4,880,000	
Oct. 28, 1911		70	5,180,000	
Oct. 31, 1911	10	74	5,580,000	
Nov. 4, 1911	10			
Nov. 6, 1911	10	57	4,620,000	No stippled cells.
Nov. 9, 1911	10	66	4,650,000	
Nov. 15, 1911	20	59	5,830,000	
Dec. 18, 1911	10	74	5,610,000	
Dec. 26, 1911	10	68	6,120,000	
Dec. 28, 1911	10	62	5,190,000	
Jan. 3, 1912		52	4,460,000	Weight, 1,665 gm. No obvious lipemia at any time.

In the majority of cases basophilic granulation of the erythrocytes was evident at one time or another. Sometimes these cells occurred in very large numbers (figure 1). This result was rather surprising as they have been generally described as an exclusive feature of toxic anemia. They were evident in the blood in some cases as early as the third day after the beginning of the bleeding, but were generally in larger numbers somewhat later. They were not especially evident in the severe grades of anemia, as large num-

bers occurred in bloods with a fairly high count. Only rarely was their presence associated with nucleated red cells. It was not uncommon, however, to find in the early stages of their appearance in the blood after hemorrhage, that the granular cells also contained a nucleus or nuclear remnant (figures 2 and 3). Commonly also the granules are very coarse and irregular (figure 4). Later, however, the granules become finer and soon disappear altogether.

TABLE II.

Rabbit 2.

Date.	Amount of blood removed, in c.c.	Per cent. of hemoglobin.	Red blood corpuscles.	Remarks.
Sept. 14, 1911	30	72	4,600,000	Weight, 2,320 gm.
Sept. 15, 1911	30	65	3,400,000	
Sept. 16, 1911	30	33	2,070,000	
Sept. 19, 1911		35	2,400,000	
Sept. 22, 1911		50	3,000,000	
Sept. 25, 1911		56	3,200,000	
Sept. 28, 1911		58	4,110,000	
Oct. 2, 1911		68	5,220,000	
Oct. 5, 1911	30	73	4,550,000	
Oct. 7, 1911	30	60	4,140,000	
Oct. 9, 1911	30			
Oct. 11, 1911	30	33	2,630,000	
Oct. 13, 1911	30	34	2,700,000	
Oct. 16, 1911	30	35	3,150,000	
Oct. 18, 1911	30	39	3,200,000	
Oct. 20, 1911	20	36	2,840,000	
Oct. 28, 1911		62	5,070,000	Weight, 2,354 gm.
Oct. 31, 1911	30	66	5,170,000	
Nov. 4, 1911	30			
Nov. 6, 1911	30	42	3,540,000	
Nov. 9, 1911	10	46	3,200,000	
Nov. 15, 1911	30	66	4,090,000	
Dec. 22, 1911	30	63	5,340,000	
Dec. 27, 1911	30	58	4,060,000	
Mar. 25, 1912	30	78	5,920,000	
Mar. 30, 1912	30	68	3,700,000	No obvious lipemia in the serum and no basophilic granulation of the erythrocytes at any time.
Apr. 6, 1912	35	72	5,300,000	

In some of these rabbits, when basophilic granular cells were present in the blood, the bleeding was discontinued and generally in a few days none could be found. In three cases they persisted for three to six weeks after discontinuing the bleeding, and in two of these animals the blood count had become practically normal.

The origin and significance of these basophilic granular erythro-

cytes have been the subject of considerable discussion. Grawitz (5) insisted that they were due to a degeneration process in the cytoplasm of the cell or to some disintegration of hemoglobin. Naegeli (6), however, denied this and considered that they were derived from nuclear destruction; and Morawitz and Blumenthal (7), Askanazy (8), Engel (9), Morris (10), Meyer and Speroni (11), Schwarz (12), and almost all the more recent authors have agreed with this. Morawitz claims, however, that they indicate essentially a regenerative process where the nucleus has been disintegrated by some toxic action.

TABLE III.

Rabbit 3.

Date.	Amount of blood removed, in c.c.	Per cent. of hemoglobin.	Red blood corpuscles.	Remarks.
Sept. 16, 1911	20	94	4,910,000	Weight, 1,255 gm.
Sept. 19, 1911	16			
Sept. 22, 1911	10	38	2,320,000	Numerous nucleated erythrocytes.
Sept. 25, 1911		30	2,710,000	
Sept. 27, 1911		36	2,860,000	
Sept. 29, 1911		35	2,460,000	
Oct. 2, 1911		43	4,280,000	
Oct. 7, 1911	20	56	4,460,000	Occasional stippled red cells.
Oct. 9, 1911		31	3,320,000	
Oct. 11, 1911	16	38	3,540,000	
Oct. 13, 1911		27	3,400,000	Only a trace of fat in the serum.
Oct. 16, 1911	20	36	3,750,000	
Oct. 18, 1911		28	2,640,000	2 per cent. of fat in the serum.
Oct. 20, 1911	20	34	2,720,000	Weight, 907 gm.
Oct. 23, 1911		18	1,850,000	6 per cent. of fat in the serum.

A somewhat similar condition has been described by Howell (13) who found, in the blood of cats after severe hemorrhage, that many of the corpuscles may contain a good sized piece of nuclear matter. He considered that on account of the rapidity with which the cells were being reproduced, this particle remained after the expulsion of the nucleus. Schmauch (14) also found what he called "endoglobuläre Körperchen" in the blood of cats after bleeding or poisoning with pyridin or bothriocephalus extract. Jolly (15) called attention to their presence in the blood of new born rats and mice, and in these animals they disappeared very soon after birth. He agreed with Schmauch that they arise from an atrophic process of the nucleus. Morris also found them in pyridin poisoning and be-

lieved them to be nuclear remnants and to represent a stage in regeneration.

The fact, however, that basophilic granular erythrocytes may appear in the blood after hemorrhage argues against their being due necessarily to a toxic degeneration of the nucleus.

The origin of this punctate basophilia opens the much disputed question of the disappearance of the nucleus of the red blood corpuscles. In erythroblastic bone marrow, the early stages of development of the red corpuscles are almost indistinguishable from those of the lymphocytes. The red cells, however, soon become larger and begin to acquire hemoglobin. In some cells there may be a large amount of hemoglobin in the cell before the nucleus disappears. Generally, however, large, pale, faintly basophilic cells without any apparent nucleus can be seen in large numbers in the blood and bone marrow, and they only subsequently acquire hemoglobin. In these latter cells the fate of the nucleus, characteristically present in the embryonal erythrocyte, can be determined fairly easily. The nucleus is not extruded, but seems gradually to lose its chromatin, and eventually disappears. In the course of this process the chromatin often becomes granular and sometimes appears to have escaped into the cytoplasm outside the nucleus. In the bone marrow, finely basophilic granular red corpuscles are not found. In the blood, however, there are apparently all stages of transition from a much more coarsely granular type, which appears early in the blood and which occurs also in erythroblastic bone marrow. In the bone marrow where new blood corpuscles are being formed rapidly, erythroblasts containing two or more nuclei are often found. One of these nuclei may be very much smaller than the other or there may be one or more small round chromatin bodies situated around the nucleus. Such cells escaping into the blood might well account for the occasional finding of basophilic granular erythrocytes which also contain a nucleus. Punctate basophilia, therefore, may be considered as representing a phase in the process of regeneration, and is not due to a toxic disintegration of the nucleus, cytoplasm, or hemoglobin.

The staining reactions of these basophilic particles in the red cells have been described by some authors as being distinct from those of

the nucleus. Using a pyronin-methyl-green stain they found that the granules might stain a violet color and the nucleus blue. This, however, does not exclude their origin from the nucleus, as in cells containing two nuclei one not infrequently stains blue and the other a reddish violet. This may be easily seen in erythroblasts containing two nuclei and is particularly well shown in liver cells, which often possess two nuclei with very different staining reactions.

Such staining methods give uncertain results, but it is interesting to find that with the aid of certain mordants, structures may be shown in normal red blood corpuscles which might well be taken for nuclei. These structures may be stained by the following method.

Fix the film for one minute in saturated aqueous mercuric chlorid solution. Wash thoroughly in water. Stain for three minutes in 1 per cent. eosin in water. Wash in water. Transfer for one minute to a saturated aqueous solution of potassium acetate. Wash in water. Mordant for five minutes in 1 per cent. phosphomolybdic acid in water. Wash thoroughly in water. Stain for three minutes in 1 per cent. methylene-blue in water.

The phosphomolybdic acid seems to act on the central part of the red cell so that the subsequent application of methylene-blue causes a deep blue staining of that part (figure 5). This stained area is apparently not due to changes in the refractiveness of the cells, nor is it due to a precipitation. The area is extremely well defined and is in the centre of the cell. The great regularity in the shape and size of these central bodies in the red cells is not altogether like that of nuclei, but if these areas do not represent nuclei, it is difficult to explain this staining reaction. In human blood the deeply staining central body occupies the greater part of the cell, and is relatively much larger than that in rabbit blood.

The resistance of the blood corpuscles to hypotonic and hypertonic salt solutions did not seem to vary much at any stage of the anemia caused by bleeding. It did not appear to be either increased or diminished. Itami and Pratt (16), however, noted after hemorrhage a slightly increased resistance, and in toxic anemia these observers found that even distilled water could be added to the red blood corpuscles without causing hemolysis.

In hemorrhagic anemia the extramedullary myeloid metaplasia is generally described as relatively slight. In the experiments of Itami,

Morawitz and Blumenthal, and Morawitz and Rehn (17), although the anemia they produced was very severe, little if any extramedullary myeloid metaplasia was observed. Certainly in the early stages of hemorrhagic anemia there is never, even in the severest degree, anything like such extensive myeloid production in the liver and spleen, as is described in anemia from toxins. Probably, however, a large proportion of this so called extramedullary myeloid production in toxic anemia represents a proliferative endothelial reaction, the majority of these new cells not developing into erythroblasts.

In three weeks in animals kept moderately anemic from hemorrhage there can generally be found numerous small clumps of myeloid cells in the liver capillaries (figure 6). Similarly, evidences of blood regeneration can also be seen in the spleen sinuses. Very extensive myeloid metaplasia may, however, be produced after a longer time. In two months, in rabbits from which twenty cubic centimeters of blood were removed three times a week, large, definite myeloid areas may be found in the liver exclusive of any inflammatory or parasitic condition. In two such rabbits these myeloid areas were numerous and large. They had even the appearances of bone marrow (figure 7). In these cases the spleen, although only slightly enlarged, showed very extensive evidences of blood regeneration. In the kidney, also, in long continued anemia from hemorrhage there may be developed numerous myeloid patches between the tubules, particularly in that part of the pyramids adjoining the cortex (figure 8). Where the animal is rapidly made extremely anemic, the regenerating power of the blood seems to become exhausted and returns to normal very slowly. In these cases of exhaustion it is not common to find any marked blood formation from extramedullary sources.

It has also been noted by Boggs and Morris (18) that lipemia is an almost constant feature of post-hemorrhagic anemia. The anemia must, however, be sufficiently severe. Fat appeared in the blood in all the rabbits where the erythrocytes fell below 2,000,000 and the hemoglobin below 30 per cent. (table III). Sometimes the proportion of fat may be enormous, giving to the serum an opaque milky appearance, from 4 to 6 per cent. being quite common in severe

anemia. In one rabbit where the blood count was very low, as much as 10 per cent. of fat was obtained from the serum. In our experiments, contrary to the results of Boggs and Morris, most of this fat could be extracted by ether, and it stained black with osmic acid. This lipemia was proportionate to the degree of anemia, and is probably related to defective oxidation in the tissues. As long as the blood count did not show a reduction of red corpuscles below 3,000,000 and the hemoglobin below 45 per cent., the animals usually gained in weight. Reduction below this, however, was accompanied by emaciation and this was sometimes very marked.

In those cases where there was lipemia it was common to find a fatty infiltration in the internal organs. The liver frequently showed extensive necrosis. The cells of the central part of the lobules became fatty and atrophied and eventually this area seemed to be composed chiefly of dilated blood-vessels (figure 9). In some cases where the anemia had been severe, this process extended almost to the periphery of the lobules, leaving only a narrow strip of normal liver tissue along the portal spaces. It seemed as if there was sufficient nutrition only for the periphery of the lobules. Fatty changes in the organs, and necrosis or fatty degeneration in the center of the liver lobules are characteristic features of pernicious anemia and have frequently been referred to as supporting the essentially toxic origin of this disease. A similar picture may, however, be produced by hemorrhage, the anemia and consequent defective oxidation and nutrition also causing extensive fatty and necrotic changes.

Pigmentation of the internal organs is necessarily much greater in the toxic anemias where large amounts of blood are destroyed in the vessels. After hemorrhage, however, there may be a considerable deposit of pigment in some of the tissues, particularly in the spleen (figure 10).

SUMMARY.

In rabbits and cats in which a hemorrhagic anemia has been produced, the hemoglobin is regenerated much more slowly than the red corpuscles, but the index, relative to the red corpuscles, never rises above one. The resistance of the corpuscles is not increased. In erythrocytes developing in the bone marrow, the nucleus of the

erythroblasts seems to lose its chromatin, but is apparently not extruded. Punctate basophilia of the erythrocytes is a common feature and seems to result from a diffusion of nuclear chromatin through the cell. Extramedullary myeloid metaplasia is generally slight, but in moderately severe and long continued anemia from bleeding it may be very extensive. Lipemia usually occurs when the blood is reduced below a certain limit. The greater part of the fat in the serum can be extracted with ether and is stained by osmic acid. Extensive fatty infiltration of the organs may occur and also widespread necrosis in the central parts of the liver lobules. Pigmentation of the internal organs is usually only slight, but may be considerable, particularly in the spleen.

Although there are minor differences, all the essential features of anemia produced by toxins can be reproduced by hemorrhage.

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EXPLANATION OF PLATES.

Plate 39.

Fig. 1. Basophilic granulation of erythrocytes, from the blood of a rabbit kept moderately anemic by repeated bleeding for two months. × 1,000.

Fig. 2. Nucleated red blood corpuscles which also contain basophilic granules in their cytoplasm. The cells are from the blood of a rabbit four days after the beginning of the bleeding. Twenty c.c. of blood had been removed daily. × 1,170.

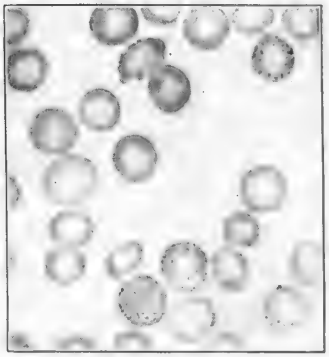

FIG. 1.

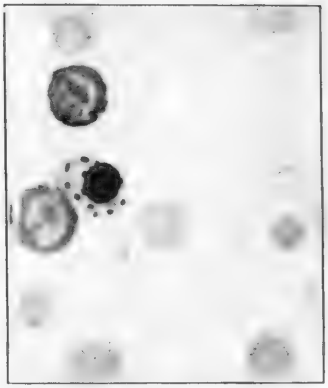

FIG. 2.

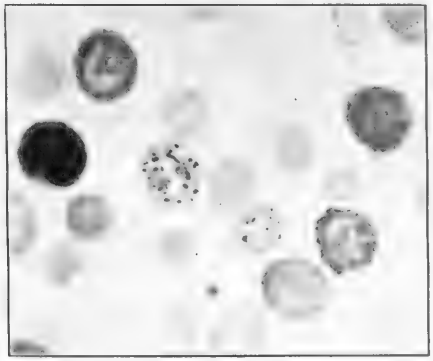

FIG. 3.

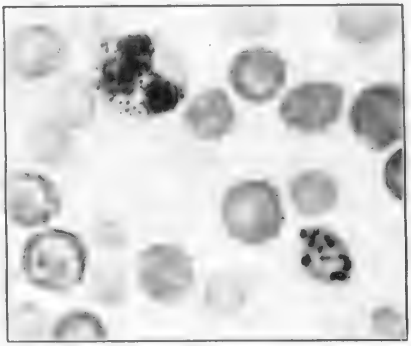

FIG. 4.

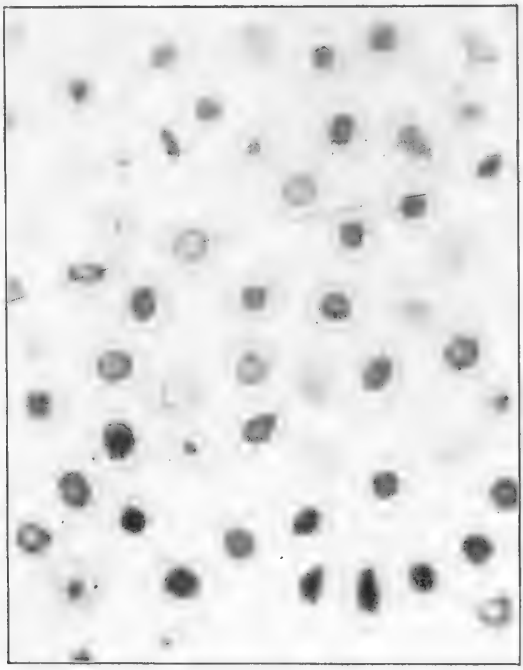

FIG. 5.

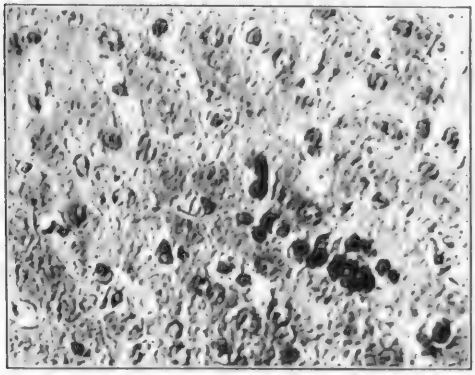

FIG. 6.

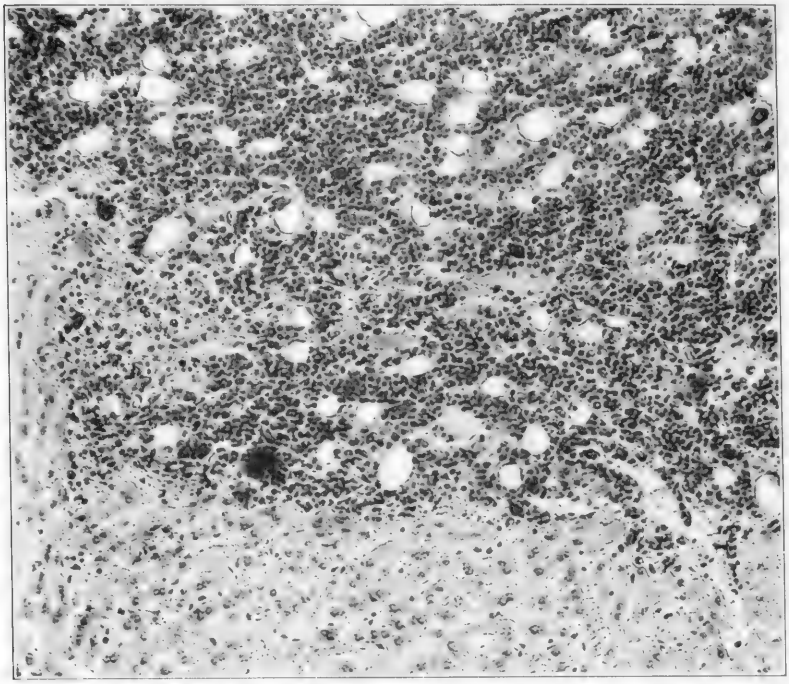

FIG. 7.

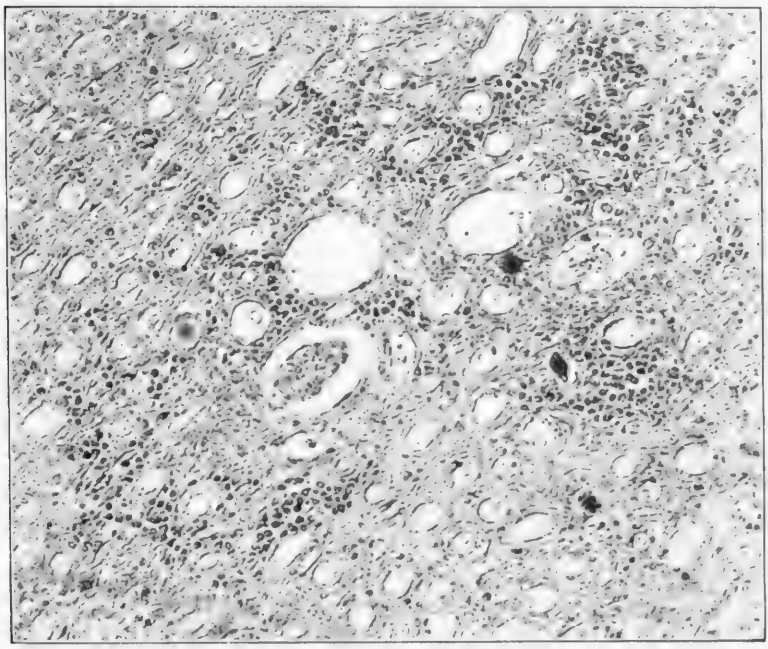

FIG. 8.

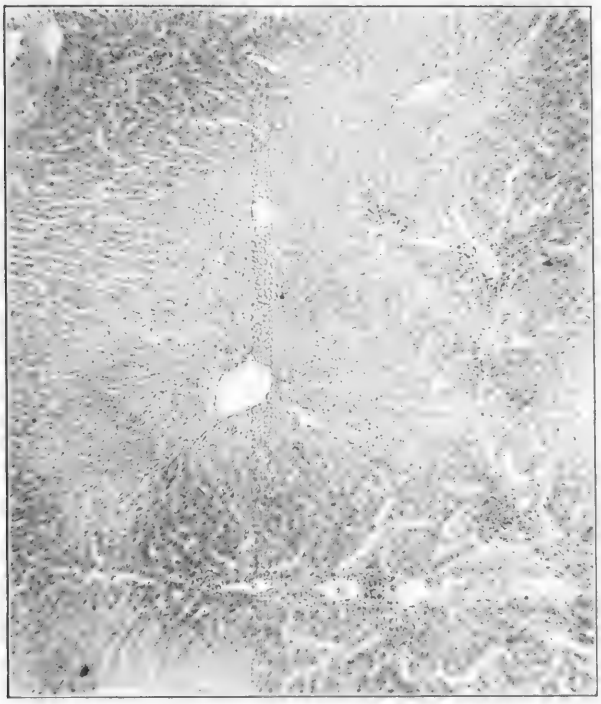

FIG. 9.

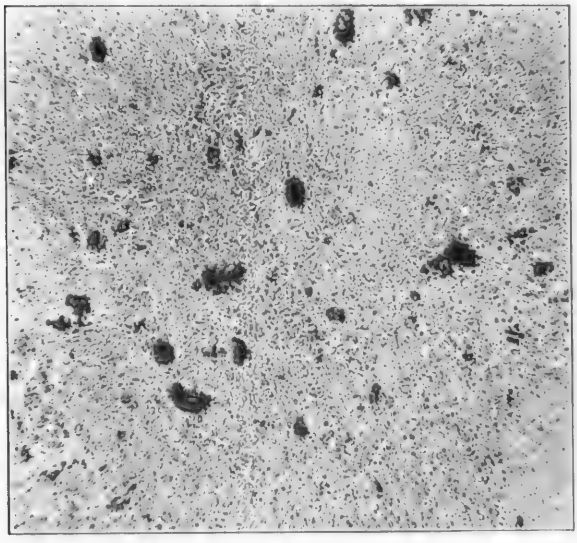

FIG. 10.

FIG. 3. Blood film from a rabbit four days after the beginning of the bleeding, 20 c.c. of blood being removed daily. Polychromatophilia and anisocytosis are very marked. Numerous erythrocytes showed basophilic granulations, which often appeared very coarse. In one of these the faint remains of a nucleus are seen. × 1,170.

FIG. 4. Blood film from a rabbit eight days after the beginning of the bleeding, 20 c.c. being removed on alternate days. One of the erythrocytes is seen to contain extremely coarse basophilic granules. × 1,170.

PLATE 40.

FIG. 5. Blood film of a normal rabbit stained by eosin-methylene-blue previously mordanted by phosphomolybdic acid. The central part of the red corpuscles is occupied by a well defined, basophilic staining structure. × 1,100.

FIG. 6. Section of the liver of a rabbit three weeks after the beginning of the bleeding, 20 c.c. of blood being removed three times a week. Some of the capillaries contain groups of developing blood cells. × 710.

PLATE 41.

FIG. 7. Section of the liver of a rabbit two months after the beginning of the bleeding, 20 c.c. of blood being removed three times a week. It shows the extensive myeloid metaplasia which may be produced by prolonged anemia from hemorrhage. Numerous large myeloid areas were found irregularly scattered throughout the liver. The spleen and kidney in this case also showed extensive evidences of blood regeneration. × 175.

FIG. 8. Section of the kidney of a rabbit that had been kept moderately anemic by bleeding for three months. Between the tubules there can be seen numerous small collections of developing blood cells, chiefly erythroblasts. × 175.

PLATE 42.

FIG. 9. Section of the liver of a rabbit showing extensive necrosis of the central portions of the liver lobules. Ten c.c. of blood were removed five times a week for one month. It was killed in apparently good condition although it had lost weight slightly, and during the last ten days there had been pronounced lipemia. × 60.

FIG. 10. Section of the spleen of a rabbit showing extensive deposition of blood pigment. Twenty c.c. of blood had been removed three times a week for six weeks. × 60.

A STUDY OF COMPLEMENT FIXATION IN SYPHILIS WITH SPIROCHÆTA CULTURE ANTIGENS.*

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It is our purpose in this communication to give the results of some work upon complement fixation in syphilitic and other sera, using specific antigens prepared from pure cultures of *Spirochæta pallida, Spirochæta pertenuis,* and *Spirochæta microdentia.* The results are compared with those obtained with the same sera and a stock antigen used in our routine Wassermann tests, this antigen consisting of an alcoholic extract of syphilitic fetal liver.

It is now recognized by most authorities that the Wassermann reaction in syphilis is not a true antigen-antibody reaction, but depends upon certain lipotropic substances produced in the tissues of syphilitics during the progress of the disease. The researches of Landsteiner, Müller, and Pötzl (1), and Porges and Meier (2) demonstrated that alcoholic extracts of normal as well as syphilitic tissues served as antigens, while those of Noguchi and Bronfenbrenner (3) proved that the active principles in the antigens used in the Wassermann test were present in the lipoidal substances found in extracts from both syphilitic and non-syphilitic tissues of man and other animals.

The success attained by Noguchi and others in obtaining *Spirochæta pallida* in pure culture gave rise to the hope that an antigen prepared from such cultures would be efficient in complement fixation in syphilis and would place the test upon the basis of a true antigen-antibody reaction. It is obvious that if such a specific antigen could be obtained the value of the Wassermann test, great

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as it is at present, would be much enhanced, as the positive reactions observed frequently in leprosy and frambesia, as well as those observed more rarely in a few other diseases, as carcinoma, malaria, and trypanosomiasis, would be eliminated. A few attempts in this direction have been made by others, but owing to the fact that the *Spirochæta pallida* has only recently been obtained in pure culture, the literature is very limited.

In 1909, Schereschewsky (4), working with impure cultures of *Spirochæta pallida,* obtained complement fixation with syphilitic sera, but he also obtained fixation with some of the control antigens. His work is unsatisfactory because the reactions occurred with the control antigen and also because of the uncertainty that exists regarding the organisms present in his mixed cultures. As one of us (Craig (5)) has shown, certain strains of staphylococci and streptococci, when growing in normal human serum, produce substances which give a positive reaction with the Wassermann test, and it is not improbable that some of the positive results obtained by Schereschewsky may have been due to lipoidal substances produced by bacteria growing in the horse serum media which he used in his cultures. At any rate, his results were suggestive and undoubtedly stimulated research along these lines.

The most important contribution to this subject is that of Noguchi (6) published in 1912, in which he details his complement fixation experiments with antigens prepared from aqueous extracts and emulsions of *Spirochæta pallida* prepared from the testicles of infected rabbits and from pure cultures. He endeavored to determine whether sera giving a positive reaction with the stock lipoidal antigen would also react with these specific antigens. He found that in certain cases of treated syphilis or in those in which the infection had existed for a very long time without symptoms, the specific antigens gave a positive reaction when the lipoidal antigen gave a negative or a very weak reaction, and that with the antigen made from a pure culture of *Spirochæta pallida* no reaction occurred in the serum from a case of leprosy, although extracts of both syphilitic and normal rabbit testicles gave a positive reaction with the same serum. Our own results with syphilitic rabbit testicle antigen will be discussed later.

Noguchi's conclusions are as follows.

1. The Wassermann reaction is caused by the lipotropic substances in sera but not by the antibodies which combine specifically with the pallida antigen.

2. The fixation produced by the culture pallida antigen with certain syphilitic sera is caused by the specific antibodies contained in the latter and may constitute a specific diagnostic method for syphilis.

3. The fixation caused by testicular extracts behaves like the culture pallida antigen in the majority of cases, but when the sera (syphilitic or leprous) contain abundant lipotropic substances, they may give a Wassermann reaction as well, which is not the case with the culture pallida antigen.

4. In the serum of rabbits with active syphilitic orchitis there is no indication of the presence of a sufficient amount of antibodies for the pallida antigen, although it gives a strong Wassermann reaction.

Noguchi regards the reaction with the pallida antigen as an index of the resistance of the patient to the infection, and states: "We have in the Wassermann reaction a fair measure of the activity of the infecting agent, and now we will have in the pallida fixation reaction a gauge for the defensive activity of the infected host."

In our work we have intentionally used alcoholic instead of watery extracts for the following reasons. (1) We desired to test the practical value of a specific antigen, and it is well known that alcoholic extracts are much more stable than watery extracts or emulsions. (2) We wished to compare our results with those obtained from our stock antigen which is an alcoholic extract. (3) It is generally recognized that an alcoholic extract of a syphilitic fetal liver is superior to that of non-syphilitic tissues because it apparently contains both the non-specific lipoids and the specific substances from the spirochætæ; hence alcohol would be suitable for the extraction of specific substances from the cultures.

As will be seen, our work shows that complement fixation in syphilis can be obtained in certain sera with an alcoholic antigen prepared from pure cultures of *Spirochæta pallida,* but it also shows that similar results can be obtained by using similar antigens prepared from cultures of other spirochætæ. We are not prepared to say definitely that these reactions are specific in the sense of being group reactions, but the evidence points in this direction. At any rate more work must be done before the use of specific culture antigens can be put on a practical basis.

Material and Technique.—In these experiments were used 51 syphilitic sera and 54 non-syphilitic sera. Of the latter 38 were from patients suffering from diseases other than syphilis, and 16 from normal individuals. In addition, 11 rabbit sera were employed, making a total of 116 sera tested. The sera were all inactivated by heating to 55 to 56° C. for one half hour just before they were tested.

The *stock antigen* was an extract of syphilitic fetal liver in ten parts of absolute alcohol, evaporated to one third of its volume, and diluted with nine parts of normal salt solution. This antigen was titrated to determine its anticomplementary qualities, and with a known syphilitic serum to determine the unit necessary to produce inhibition of hemolysis.

The *culture antigens* were prepared from cultures obtained through the kindness of Dr. Noguchi, pure cultures of the spirochætæ being grown on the media recommended by him, consisting of one part of ascitic fluid and two parts of a weakly alkaline agar to which a piece of sterile rabbit kidney was added. Of each spirochæta, two tubes containing rich growths, of one and two months, respectively, were selected. The oil was poured off and the tube filed and broken. The agar column was removed, the upper or uninfected portion was cut away, and the tissue picked out. The remaining medium was shaken up with ten times its weight of absolute alcohol and was extracted for ten days, being shaken frequently. The extract was then filtered, the filtrate was evaporated to one third its volume, and was titrated in a dilution of 1 to 10 with normal salt solution. The antigens thus obtained were all titrated to determine their anticomplementary qualities and, in addition, the pallida antigen was titrated against a positive syphilitic serum.

All the antigens in this series of experiments, including the stock antigen, were used in a dose of 0.1 of a cubic centimeter of a 1 to 10 dilution.

For complement fresh guinea pig serum was used, while for amboceptor the serum of rabbits immunized to human red blood corpuscles was employed. The blood suspension consisted of a 1 per cent. suspension of human red blood corpuscles in normal salt solution.

As a control on the extract of cultures of the spirochæta, an alcoholic extract of the media employed in growing the spirochætæ was prepared in the same manner. These media, of course, had not been used for cultivation purposes.

Results with Normal Sera.—Sera from sixteen normal individuals were tested with the stock antigen, and with pallida, pertenuis, and microdentia antigen, but all of them gave negative results. In not a single instance was there the least trace of a reaction in these sera. It is therefore evident that none of these antigens react with normal blood serum. In each test 0.8 of a cubic centimeter of inactivated serum was used.

Results with Serum from Diseases Other than Syphilis.—Sera from thirty-eight patients suffering from diseases other than syphilis

were tested, a negative result being obtained in all but one case; this was diagnosed as arthritis and gave a plus result (i. e., an inhibition of at least 50 per cent.) with the microdentia antigen. This one positive result prevents our stating that none of these antigens react with diseases other than syphilis, but it is our opinion that it will be found that a positive reaction will not be obtained in non-syphilitic diseases, and that this one positive reaction was due to some accidental cause, perhaps to the contamination of the serum with bacteria. In each test 0.8 of a cubic centimeter of inactivated serum was used.

Results with Serum from Syphilitic Patients.—Fifty-one sera from as many syphilitic patients were tested, 0.8 of a cubic centimeter of the inactivated serum being used in each test. In recording these tests the sign ++ means absolute inhibition of hemolysis; the sign +, at least 50 per cent. of inhibition; and the sign +—, weaker degrees of inhibition. The sign — means total lack of inhibition, while the sign 0 means that no test was made. The results of the tests with syphilitic sera are shown in table I.

An analysis of table I is interesting. No positive results were obtained with the antigen from the uninoculated culture, a fact which proves that there is nothing in the medium used for cultivation which produces the reaction. A large percentage of the sera that gave positive results with the stock antigen also reacted with the pallida antigen, no less than twenty-seven of the thirty-six positive cases, or 75 per cent., reacting with both antigens; although, as a rule, the reactions were weaker with the pallida than with the stock antigen. A large percentage of sera that were positive with the stock antigen were also positive with the pertenuis and microdentia antigen.

An interesting point is that while in general the reactions were weaker with the specific antigens than with the stock antigen, there occurred several instances in which the reactions with the specific antigens were stronger. Thus with sera 11 and 19 the stock antigen gave a plus-minus reaction, while the pallida antigen gave a double plus reaction; while in sera 22 and 29 the stock antigen gave a plus-minus and the pallida antigen gave a plus reaction. In serum

38 the stock antigen gave a negative reaction while the pallida gave a plus reaction.

TABLE I.

Complement Fixation with Stock Antigen and Specific Spirochæta Antigens in Human Syphilitic Sera.

Serum No.	Stock antigen.	*S. pallida* antigen.	*S. pertenuis* antigen.	*S. microdentia* antigen.	Control antigen.	Remarks.
1	++	++	o	o	−	Secondary, early.
2	++	+	o	o	−	Primary.
3	++	+−	+−	o	−	Secondary; 2 doses salvarsan.
4	++	−	o	o	o	Latent, 18 yrs.; 4 doses salvarsan.
5	++	+	+	+	−	Latent, 4 yrs.; 2 doses salvarsan.
6	++	−	+−	−	−	Latent, 3 yrs.; 3 doses salvarsan.
7	++	−	−	o	−	Tertiary, 10 yrs.
8	++	−	o	o	−	Secondary, 9 mos.
9	+	+	o	o	o	Secondary, early.
10	+	+	o	o	−	Primary.
11	+	++	o	o	−	Primary, 2 wks.
12	+	+	o	o	o	Latent, 2 yrs.
13	+	−	o	+−	o	Primary, 2 wks.
14	+	−	o	+	−	Primary, 4 wks.
15	+	+	o	+	−	Secondary, early.
16	+	+	−	−	−	Secondary, 1 yr.
17	+	−	o	+	−	Tertiary.
18	+	+	+−	+	−	Secondary.
19	+	++	o	+	−	Tertiary; duration unknown.
20	+	+−	+−	+−	−	Latent; duration unknown.
21	+−	+−	o	o	−	Latent, 3 yrs.
22	+−	+	o	o	−	Primary, 3 wks.
23	+−	+−	o	o	o	Secondary, early.
24	+−	−	−	−	−	Primary, 2 wks.
25	+−	−	−	o	−	Epilepsy.
26	+−	+−	o	+−	−	Arthritis, syphilitic.
27	+−	+−	o	+−	o	Latent, 2 yrs.
28	+−	+−	o	+−	−	Latent, 2 yrs.
29	+−	+	o	+	−	Latent, 1 yr.
30	+−	+−	−	+−	−	Secondary, 1 yr.
31	+−	++	++	+	−	Latent; duration unknown.
32	+−	+−	o	+	−	Latent, 3 yrs.
33	+−	+−	+−	−	−	Secondary, 3 mos.
34	+−	+	+−	+−	−	Latent, 4 yrs.
35	+−	+−	−	+−	−	Latent, 6 yrs.
36	+−	+−	+−	−	−	Secondary, early.
37	−	+−	−	+−	−	Latent, 2 yrs.
38	−	+	+	+−	−	Latent, 1½ yrs.; 4 doses salvarsan.
39	−	+−	−	−	−	Latent, 6 yrs.
40	−	+−	−	o	o	Primary, 2 wks.

Each serum was also tested without antigen, as is usual in the Wassermann test.

As regards the reactions given with the antigen prepared from pure cultures of *Spirochæta pallida,* seventeen sera gave the same result as with the stock antigen, four gave weaker reactions, six gave stronger reactions, and four gave positive results in sera in which the stock antigen gave negative reactions. However, the large proportion of negative reactions (seven out of twenty) in sera giving a double plus or plus reaction with the stock antigen demonstrates the superiority of the latter antigen in the diagnosis of syphilis.

Of the four sera giving a negative reaction with the stock antigen, one gave a plus reaction with the pallida antigen while three gave a plus-minus reaction with the same antigen. All these cases had been treated and the one giving a plus reaction with the pallida antigen had previously given a double plus reaction with the stock antigen, but later had been given four doses of salvarsan.

There were eighteen sera tested with the antigen prepared from pure cultures of *Spirochæta pertenuis.* Of these eight gave a negative result; one in a serum giving a plus reaction with the stock antigen, four in sera giving a plus-minus reaction with the stock antigen, and three in sera giving a negative reaction with that antigen. In general the results obtained with the pertenuis antigen were similar to those obtained with the pallida antigen.

There were twenty-five sera tested with the antigen prepared from pure cultures of *Spirochæta microdentia.* Of these only six gave a negative reaction. Of the negative sera, five gave reactions with the stock antigen, although in three of them the reaction was only a plus-minus one. Of the positive sera, eleven gave the same results with the microdentia antigen as with the stock antigen, while in three sera the reaction with the microdentia was stronger than with the stock antigen. In three sera the reaction with this antigen was weaker than with the stock antigen.

Fifteen of the sera were tested with all the antigens, and the results are shown in table II. They are of interest because they strongly suggest the group nature of the reaction.

In four of the sera the results were the same with all the culture antigens, but in the remainder they varied, the pallida antigen

giving reactions more nearly like those obtained with the stock antigen, while the results obtained with the microdentia and pertenuis were strikingly similar, although in its results the microdentia antigen approached the pallida nearer than did the pertenuis.

TABLE II.

Complement Fixation in Fifteen Syphilitic Sera Tested with All the Antigens.

Serum No.	Stock antigen.	*S. pallida* antigen.	*S. pertenuis* antigen.	*S. microdentia* antigen.	Control antigen.	Remarks.
1	++	+	+	+	−	Latent, 4 yrs.
2	++	−	−	−	−	Latent, 3 yrs.
3	+	+−	+−	+−	−	Latent; duration unknown.
4	+−	−	−	−	−	Primary, 2 wks.
5	+	+	−	−	−	Secondary, 1 yr.
6	+	+	+−	+	−	Secondary; duration unknown.
7	+−	+−	−	+−	−	Secondary, 1 yr.
8	+−	++	++	+	−	Latent; duration unknown.
9	+−	+−	+−	−	−	Secondary, 3 mos.
10	+−	+	+−	+−	−	Latent, 4 yrs.
11	+−	+−	−	+−	−	Latent, 6 yrs.
12	−	+−	−	+−	−	Latent, 2 yrs.
13	−	+	+	+−	−	Latent, 1½ yrs.
14	−	+−	−	−	−	Latent, 6 yrs.
15	−	+−	−	o	o	Primary, 2 wks.

Each serum was also tested without antigen, as is usual in the Wassermann test.

Results Obtained in the Various Stages of Syphilis.—Table III gives the results obtained with the different antigens in the various stages of syphilis, and while the cases are, perhaps, too few in number to justify any radical conclusions, it is evident that the culture antigens give reactions in every stage of the disease, although more positive reactions are obtained in latent cases.

The occurrence of the reaction with the culture antigens in the primary stage of syphilis is of importance, for if we believe that these are true antigen-antibody reactions, it must be admitted that the antibodies occur in the patient's serum very early in the disease, for in no less than five of the thirteen sera from patients in the primary stage of the disease, the pallida antigen gave a positive reaction. In the secondary stage ten of the thirteen sera tested gave positive reactions with the pallida antigen, and in the latent stage (including both early and late latent cases) fifteen of the twenty-one sera gave positive reactions.

TABLE III.

Complement Fixation in Various Stages of Syphilis with Stock Antigen and Specific Spirochæta Antigens.

	Serum No.	Stock antigen.	*S. pallida* antigen.	*S. pertenuis* antigen.	*S. microdentia* antigen.	Control antigen.	Remarks.
Primary stage.	1	++	+	o	o	−	Duration 3 wks.
	2	+	+	o	o	−	Duration 2 wks.
	3	+	++	o	o	−	Duration 2 wks.
	4	+	−	o	+−	o	Duration 2 wks.
	5	+	−	o	+	−	Duration 4 wks.
	6	+−	+	o	o	−	Duration 3 wks.
	7	+−	−	−	−	−	Duration 2 wks.
	8	−	+−	−	o	o	Duration 2 wks.
	9	−	−	o	o	−	Duration 10 days.
	10	−	−	o	−	−	Had salvarsan.
	11	−	−	−	−	−	Duration 8 days. Had salvarsan.
	12	−	−	o	−	o	Duration 5 days. Had salvarsan.
Secondary stage.	1	++	++	o	o	−	Early.
	2	++	+−	+−	o	−	2 doses salvarsan.
	3	++	−	o	o	−	Duration 9 mos.
	4	+	+	o	o	o	Early.
	5	+	+	o	+	−	Early.
	6	+	+	−	−	−	Duration 1 yr.
	7	+	+	+−	+	−	Early.
	8	+−	+−	o	o	o	Early.
	9	+−	+−	−	+−	−	Duration 1 yr.
	10	+−	+−	+−	−	−	Duration 3 mos.
	11	+−	+−	+−	−	−	Early.
	12	−	−	−	o	o	2 doses salvarsan.
	13	−	−	o	−	−	Duration 2 mos.
Tertiary stage.	1	++	−	o	o	−	Duration 10 yrs.
	2	+	−	o	+	−	Duration unknown.
	3	+	++	o	+	−	Duration unknown.
	4	−	−	o	−	o	1 dose salvarsan.
	5	−	−	−	−	−	Duration unknown.
Latent stage.	1	++	+−	o	o	o	Duration 18 yrs.
	2	++	+	+	+	−	Duration 4 yrs.
	3	++	−	−	−	−	Duration 3 yrs.
	4	+	+	o	o	o	Duration 2 yrs.
	5	+	+−	+−	+−	−	Duration unknown.
	6	+−	+−	o	o	−	Duration 3 years.
	7	+−	+−	o	+−	o	Duration 2 yrs.
	8	+−	o	+−	+−	−	Duration 2 yrs.
	9	+−	+	o	+	−	Duration 1 yr.
	10	+−	++	++	+	−	Duration unknown.
	11	+−	+−	o	+	−	Duration 3 yrs.
	12	+−	+	+−	+−	−	Duration 4 yrs.
	13	+−	+−	−	+−	−	Duration 6 yrs.
	14	−	+−	−	+−	−	Duration 2 yrs.
	15	−	+	+	+−	−	Duration 1½ yrs.
	16	−	+−	−	−	−	Duration 6 yrs.
	17	−	−	o	−	o	Duration 2 yrs.
	18	−	−	o	+−	o	Duration 3 yrs.
	19	−	+−	−	+−	−	Duration 6 yrs.
	20	−	−	−	o	o	Duration 3 yrs. Had salvarsan.
	21	−	−	−	o	o	Duration 6 yrs. Had salvarsan.

The highest percentage of reactions with the pallida antigen occurred in the secondary stage of syphilis. The patients from whom the serum was obtained, with two exceptions, presented active lesions of the disease.

Results with Rabbit Serum.—We have used the serum from a rabbit suffering from a syphilitic orchitis; the serum from three rabbits that had recovered from a syphilitic orchitis; the serum from two that had recovered from frambesia; and the serum from four normal rabbits. Table IV gives our results in these tests.

TABLE IV.

Complement Fixation with Stock Antigen and Specific Spirochæta Antigens in Rabbit Sera.

Rabbit No.	Stock antigen.	*S. pallida* antigen.	*S. pertennis* antigen.	*S. microdentia* antigen.	Control antigen.	Remarks.
79 (1)	++	–	–	–	–	Active syphilitic lesions.
79 (2)	++	–	–	–	–	Active syphilitic lesions.
77 (1)	–	–	–	–	o	Recovered from syphilis.
77 (2)	–	–	–	–	o	Recovered from syphilis.
75	–	–	–	–	–	Recovered from syphilis.
85 (1)	–	–	–	o	–	Recovered from yaws.
85 (2)	–	–	–	–	–	Recovered from yaws.
1	–	–	–	o	–	Normal rabbit.
2	–	–	–	o	–	Normal rabbit.
84	–	–	–	o	–	Normal rabbit.
86	–	–	–	o	o	Normal rabbit.

Each serum was also tested without antigen, as is usual in the Wassermann test. The quantity of rabbit serum used was 0.1 and 0.2 c.c., inactivated.

The important fact shown by this table is that no reactions occurred in any of the sera except in rabbit 79, presenting an active syphilitic orchitis, and that this serum was positive only with the stock antigen. In this respect our results confirm those of Noguchi, who also found that his pallida antigen did not react with rabbit serum even though the animal presented active lesions of the infection.

Experiments with Rabbit Testicle Antigen.—Over a year ago we experimented with alcoholic antigens made from the testicles of rabbits suffering from syphilitic orchitis and yaws, and our results were published by Nichols (7). We found that while such antigens gave positive results with certain syphilitic sera, they also reacted

with certain normal sera, as well as with sera from other diseases. In addition we found that the antigen prepared from the testicles of rabbits inoculated with syphilis gave positive results with the sera of rabbits inoculated with yaws, and *vice versa.* As we found that antigens prepared from normal rabbit testicles gave similar results we concluded that these reactions were in no sense specific.

Discussion of Results.—The results obtained with the pallida antigen approach closely those obtained with the stock antigen, but were generally weaker, and in some undoubted syphilitic cases, where the stock antigen gave strong reactions, the pallida antigen gave a negative result. It is evident that the pallida antigen gives more uniform results than either of the others, but there is no great difference between them in the majority of the sera tested.

The results obtained with the microdentia antigen are almost identical with those obtained with the pallida antigen, a rather interesting observation when one remembers that this spirochæta resembles *Spirochæta pallida* more closely morphologically than any other so far described, except *Spirochæta pertenuis.* It is not surprising that the pallida and pertenuis should both give reactions, but the reaction of the microdentia is more difficult to explain, as this spirochæta is not pathogenic for animals.

The large number of positive reactions in secondary cases showing active lesions is difficult to reconcile with the theory that complement fixation in syphilis with specific antigens is a gauge of the resistance of the patient, if one accepts absence of visible symptoms as indicating resistance.

In considering the results given above, the origin and history of the cultures naturally come in question. The pallida strain used was grown by Noguchi from the infected testicle of a rabbit and came originally from mucous patches of a marked secondary case of syphilis. The culture was pathogenic for rabbits soon after inoculation. The pertenuis strain was also grown from the testicle of a rabbit after passage through a monkey, and came originally from a characteristic case of yaws in a colored soldier returning from the Philippines. The microdentia strain was isolated by Noguchi from peridental material in a child. This strain was not pathogenic for animals. It produces a distinctive odor of putrefaction in cul-

tures, and it is morphologically distinctly finer than either the pallida or the pertenuis. There is no doubt in our minds regarding the identity of the cultures.

It seems to us that these reactions with specific antigens may be explained in either of the following ways:

1. That they are non-specific reactions and are due to lipoid substances produced in the media during the growth of the organisms. The failure to react with normal sera and rabbit sera speaks against this conclusion, but the point can be settled by further work on other organisms such as refringens, vibrios, and bacteria.

2. That they are specific group reactions. It is not surprising if some group reaction exists between the pallida, the pertenuis, and even the microdentia, for the microdentia, while in other respects much farther removed from the pallida than the pertenuis, resembles the pallida more closely morphologically than any other spirochæta. The specific action of salvarsan upon these spirochætæ also argues some connection between them.

It is believed that these experiments suggest that the complement fixation reaction obtained in syphilis with antigens made from pure cultures of the spirochætæ is a group reaction, for it is very difficult to explain the results in any other way. Further work should be done with spirochætæ farther removed from the pallida (e. g., *Spirochæta recurrentis* and *Spirochæta refringens*), and with various species of bacteria.

As regards the practical value of alcoholic extracts of pure cultures of *Spirochæta pallida* in the diagnosis of syphilis, it is evident from our results that they cannot be depended upon, for many cases of undoubted syphilis, giving a double plus or plus reaction with our stock syphilitic liver antigen, gave absolutely negative results with the culture antigens. For diagnostic purposes, therefore, the alcoholic extracts of pure cultures of *Spirochæta pallida,* instead of possessing advantages over an alcoholic extract of fetal syphilitic liver, possess a much weaker complement fixation power and a negative reaction is often encountered in patients giving a positive reaction with the Wassermann test.

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THE RETENTION OF FOREIGN PROTEIN BY THE KIDNEY.

A Study in Anaphylaxis.*

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This investigation was undertaken for the purpose of obtaining exact information in regard to two points of prime importance in connection with the physiology and the pathology of the kidney: (1) does a foreign protein tend to accumulate in the kidney; and (2) if so, is this tendency greater in the presence of a nephritis? The first problem is of importance mainly in connection with the etiology of nephritis; the second, in connection with the prognosis, treatment, and dietetics of nephritis.

It is obvious that the acute nephritides associated with acute infectious diseases (scarlet fever, diphtheria, etc.) are due either to the poison elaborated by the causal microörganism or to the products of tissue catabolism. Soluble bacterial and protozoan poisons as well as the poisonous products of tissue destruction act on the kidney cells, either in a specific selective way or through irritation as the result of long continued contact during a process of gradual elimination.

The latter has quite generally been assumed to be the correct explanation, but this assumption has no experimental or other data as a basis. Because the glomerulus is the usual seat of the renal lesion of scarlet fever, it is believed that the glomerulus is affected as the result of the elimination by the kidney of the toxins of scarlet fever. So also in the case of cholera and eclampsia, the epithelial lesion in the kidney tubules is supposed to be due to the elimination of the toxins of these diseases through the cells of the

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tubules. Likewise, in the experimental nephritides, the glomerular lesion due to arsenic and the tubular lesions due to potassium chromate are similarly explained.

On the other hand, it is possible that these various poisons may have a specific selective affinity for certain constituents of the various cells of the kidney, and may be "fixed" as it were in these cells, remaining within them appreciably longer than the period usually required for mere elimination.

The solution of this problem depends, therefore, on some method capable of demonstrating that a foreign protein introduced into the blood-stream is (1) equally distributed in the blood and organs; or (2) that it is present in greater amount in the kidney than in the blood; or (3) that it may be demonstrated in the kidney after it has disappeared from the circulating blood. These various possibilities may be tested by a method that is dependent on the phenomenon of anaphylaxis, and the suggestion that these problems could be solved by this method I owe to the papers of Vaughan and his associates[1] on the parenteral introduction of protein, and to the statement of these investigators that a foreign protein (egg-white) may be detected in the kidney of the rabbit after it has disappeared from the circulating blood.

Previously Wells had used the anaphylaxis reaction in the study of alimentary albuminuria, and more recently Van Alstyne and Grant have used it to demonstrate the absorption of albumin from the intestine without digestion. Wells found that guinea pigs can not be sensitized to egg-albumin with the urine of individuals receiving a diet of raw eggs, even when such individuals have an alimentary albuminuria, and concluded that ingested egg-albumin does not pass through the body unchanged, and that the albuminuria under such circumstances is due to the elimination, not of ingested foreign protein, but of serum proteins.

The use of the anaphylaxis reaction to detect foreign protein in the circulating blood or in the organs has been attempted, so far as

[1] Vaughan, V. C., Cumming, J. G., and McGlumphy, C. B., The Parenteral Introduction of Proteins, *Ztschr. f. Immunitätsforsch., Orig.*, 1911, ix, 16; Vaughan, V. C., Cumming, J. G., and Wright, J. H., Protein Fever, *ibid.*, 458.

I am aware, only by Vaughan and his associates, and by Van Alstyne and Grant.[2]

The latter injected into a vein of a dog 20 c.c. of a mixture of equal parts of egg-albumin and salt solution, and drew blood after one quarter of an hour, and after four, forty-eight, and seventy-two hours. The serum of each of the various samples and the urine collected during the first twenty-four hours were used to sensitize guinea pigs. At a later period an intoxicating injection of egg-albumin caused in each animal the characteristic symptoms of anaphylaxis, these being least marked in the animal sensitized with the serum drawn after seventy-two hours, only moderately severe in the animal receiving the forty-eight hour serum, but severe in all of the others, including the animal that received the urine.

In a second series of experiments Thiry-Vella fistulas were made at various levels of the intestinal canal, egg-albumin was placed in these, and blood was drawn at periods varying from one to three hours. The blood serum thus obtained was injected into guinea pigs and sensitized these animals to egg-albumin. Similar results were obtained with the urine of the first twenty-four hours.

The experiments of Vaughan and his associates may be divided into two groups. In the earlier experiments they used for the sensitization of guinea pigs the blood of rabbits obtained a few hours after the feeding or injection of egg-albumin.

In one experiment they found that one and a half hours after the intravenous injection of egg-albumin (50 c.c. of 1:1 solution) the blood failed to sensitize guinea pigs; in a second, failure was noted after two hours; and in a third, after two and a half hours.

In a fourth experiment blood drawn one hour after the injection of egg-albumin sensitized, while that drawn after two, three, and five hours failed to sensitize; on the other hand, all the extracts of brain, kidney, spleen, and liver, made six hours after the injection of egg-white, possessed a sensitizing power which was most marked in the extracts of the kidney and liver.

In a fifth experiment after the egg-white had disappeared from the circulating blood the animal was transfused with one liter of salt solution and samples of fluid were taken from the heart in amounts of 2 c.c. These had the power to sensitize as did also extracts of liver, spleen, and muscle; extracts of brain and kidney failed to sensitize.

In a later communication it was shown that extracts of skin, kidney, brain, liver, spleen, intestine, and stomach from rabbits killed twenty-four and forty-eight hours after intravenous injection of egg-albumin are capable of sensitizing guinea pigs, but that similar extracts from animals killed after seventy-two hours do not have this power. The symptoms of anaphylaxis are, the writers note, most marked in guinea pigs receiving extracts of kidney and spleen.

It was this difference between the results at forty-eight and seventy-two hours and the marked reaction in guinea pigs receiv-

[2] Van Alstyne, E. V. N., and Grant, P. A., The Absorption of Albumin without Digestion, *Jour. Med. Research,* 1911, xxv, 399.

ing extracts of the kidney that suggested the use of this method of sensitization for the purposes of the present investigation.

METHODS.

The usual procedure has been to inject intravenously a group of three, four, or five rabbits with a foreign protein, to chloroform the animals at intervals of twenty-four hours, and then to inject their blood or the extracts of their organs into the peritoneal cavity of guinea pigs. After two or three weeks these guinea pigs received either intravenously or intraperitoneally an injection of the same kind of protein originally injected into the rabbits.

Egg-albumin and horse serum have been the substances used in most of the experiments. The egg-white has been diluted with three parts of physiological salt solution, except in some of the earlier experiments in which a dilution of equal parts was tried; but as this dilution frequently caused sudden death, it was abandoned for the dilution of one part to three. With the latter dilution no immediate ill effects have been seen, though very rarely an animal died on the second or third day after the injection.

All injections of egg-white and serum were made in the ear vein, forty cubic centimeters of diluted egg-albumin (ten cubic centimeters of egg-white) and ten cubic centimeters of undiluted horse serum being the amounts injected. All animals were killed by chloroform except when it was necessary to obtain the blood or to wash the organs with salt solution. Under the latter circumstances, ether anesthesia was always used. Extracts were made by grinding the organs in a mortar with sand, adding twenty cubic centimeters of salt solution, and allowing the mixture to stand at 33° to 35° C. for eighteen to twenty-four hours. The fluid was then removed by centrifugalization. The extracts thus obtained were injected into guinea pigs in amounts of five to eight cubic centimeters, and defibrinated blood in amounts of five cubic centimeters. All extracts for sensitization were injected intraperitoneally.

The intoxicating dose was given in the early experiments both intraperitoneally and intravenously; in the later work, injections were given into the jugular vein only. The amount of egg-white used for an intraperitoneal injection was five cubic centimeters of a 50 per cent. (1 to 1) dilution, and for intravenous injection four

cubic centimeters of a 25 per cent. (1 to 3) dilution. Horse serum was given by these two methods in doses of five cubic centimeters and one to two cubic centimeters respectively.

The varying degrees of sensitization, as presented in the tables, are indicated by the following signs:

+ + + + = acute fulminant anaphylaxis with death and typically insufflated lungs at autopsy.
+ + + = marked symptoms,—severe respiratory distress, "bucking," convulsions, and prostration, but with eventual recovery.
+ + = moderate attack,—spasmodic cough, respiratory distress, "bucking," and a tendency to lie prone.
+ = "scratching," cough, slight "bucking," slight respiratory distress.
o = no effect.

The following table confirms Vaughan's single observation as to the presence of egg-albumin in the kidney twenty-four and forty-eight hours after injection, and its absence (or failure to sensitize) after seventy-two hours; it also shows the results of control experiments.

SERIES I.

Egg-White.

The rabbits were killed one, two, three, and four days after intravenous injection of 10 c.c. of egg-white diluted with salt solution. Guinea pigs received intraperitoneally extracts of the kidneys of each rabbit, and after three weeks had elapsed they received intraperitoneally an intoxicating dose of 5 c.c. of a mixture of equal parts of egg-white and salt solution, or intravenously 4 c.c. of a 1 : 3 dilution.

No. of animal.	Extract.	Method of injection.	Result.
Guinea pig 1	24 hrs.	Intraperitoneal.	+++
Guinea pig 2	24 hrs.	Intravenous.	++++
Guinea pig 3	48 hrs.	Intraperitoneal.	+
Guinea pig 4	48 hrs.	Intravenous.	++
Guinea pig 5	72 hrs.	Intraperitoneal.	o
Guinea pig 6	72 hrs.	Intravenous.	o
Guinea pig 7	96 hrs.	Intraperitoneal.	o
Guinea pig 8	96 hrs.	Intravenous.	o
Control 1[3]		Intraperitoneal.	o
Control 1		Intravenous.	o
Control 2[3]		Intraperitoneal.	o
Control 2		Intravenous.	o
Control 3[3]		Intravenous.	++++

[3] Control 1. Normal guinea pigs.

Control 2. Guinea pigs that had received, twenty-eight days before, extracts of normal rabbit kidney.

Control 3. A guinea pig that had received, three weeks before, an extract prepared from normal rabbit kidney and 1 c.c. of egg-white.

SERIES II.

Horse Serum.

This series is like series I except that horse serum was used instead of egg-white. The rabbits were killed one, two, three, and four days after receiving intravenously 10 c.c. of normal horse serum. Extracts of the kidneys of each animal were injected intraperitoneally into guinea pigs, and after three weeks each guinea pig received intravenously 2 c.c. of the same horse serum, or 5 c.c. intraperitoneally.

No. of animal.	Extract.	Method of injection.	Result.
Guinea pig 1	24 hrs.	Intraperitoneal.	+++
Guinea pig 2	24 hrs.	Intravenous.	++++
Guinea pig 3	48 hrs.	Intraperitoneal.	++
Guinea pig 4	48 hrs.	Intravenous.	+++
Guinea pig 5	72 hrs.	Intraperitoneal.	++
Guinea pig 6	72 hrs.	Intravenous.	++++
Guinea pig 7	96 hrs.	Intravenous.	0
Control 1[4]		Intravenous.	0
Control 2[4]		Intravenous.	0
Control 3[4]		Intravenous.	++++

In series II the kidney extract of the third day still retained power to sensitize.

At this point, it seemed desirable to determine whether bacterial proteins were retained by the kidney in a form capable of sensitizing guinea pigs. The work of others on sensitization to tuberculin and to pneumococcus protein offered some hope of successful results, and, moreover, the use of bacterial proteins, which are factors in the production of nephritis, would, it was obvious, allow more definite conclusions concerning their possible retention in man, than would studies with egg-albumin and horse serum.

Rabbits were therefore given intravenously tuberculin in the form of O. T. bovine,[5] a tuberculin[6] prepared without heating, mallein,[7] mallease,[8] and pneumococcus protein.[9] A vegetable protein, gliadin,[10] was also used.

[4] Control 1. Normal guinea pig.

Control 2. A guinea pig that had received, three weeks before, an extract of normal rabbit kidney.

Control 3. A guinea pig that had received, three weeks before, an extract prepared from normal rabbit kidney and 1 c.c. of horse serum.

[5] Products of the Pennsylvania Live Stock Sanitary Board, procured through Dr. John Reichel.

[6] Procured from Dr. John Reichel of H. K. Mulford and Co.

Extracts of the kidneys of rabbits which had received respectively twenty-five cubic centimeters of a 2 per cent. solution of gliadin in 0.1 per cent. sodium hydrate, the protein of six billion pneumococci, ten cubic centimeters of tuberculin, and five cubic centimeters each of mallein and mallease, all failed to sensitize guinea pigs to the respective proteins.

The disappointing results of these experiments necessitated a return to the use of egg-albumin and horse serum as the most satisfactory agents to be used in settling the fundamental points of the investigation. The most important of these points was to determine the distribution of the egg-white in the body of the injected rabbit. Does a foreign protein tend to accumulate to a greater extent in the kidney than in other organs, or is it present in the kidney in the same concentration as in other organs; in other words, is the content of foreign protein of an organ dependent merely upon the amount of the circulating blood present in that organ? This point was determined by the experiments summarized in series III and IV, representing the injection of egg-albumin and horse serum respectively.

SERIES III.

Comparison of Sensitizing Power of Blood, Liver, and Kidney after the Intravenous Injection of Egg-White.

Rabbits received 10 c.c. of egg-white diluted with 30 c.c. of salt solution, and were killed after twenty-four, forty-eight, and seventy-two hours, respectively. Blood and extracts of liver and kidneys were injected into guinea pigs. After three weeks each guinea pig received intravenously 4 c.c. of a 1:3 egg-white dilution or intraperitoneally 5 c.c. of a 1:1 dilution.

Guinea pigs.	Time.	Blood.	Liver.	Kidney.	Mode of injection of intoxicating dose.
Group 1	24 hrs.	0	+	++	Intraperitoneal.
Group 2	24 hrs.	++	+++	+	Intravenous.
Group 3	48 hrs.	+++	+	0	Intraperitoneal.
Group 4	48 hrs.	+	++	0	Intravenous.
Group 5	72 hrs.	0	0	0	Intraperitoneal.
Group 6	72 hrs.	0	0	0	Intravenous.

[7] Products of the Pennsylvania Live Stock Sanitary Board, procured through Dr. John Reichel.

[8] Procured from Dr. John Reichel of H. K. Mulford and Co.

[9] Prepared by Dr. J. A. Kolmer according to the method of E. C. Rosenow, Pneumococcus Anaphylaxis and Immunity, *Jour. Infect. Dis.*, 1911, ix, 190.

[10] Product prepared by Drs. T. B. Osborne and H. G. Wells, and obtained through the latter.

SERIES IV.

The same as series III except that horse serum (10 c.c.) was used instead of egg-white.

Guinea pigs.	Time.	Blood.	Liver.	Kidney.	Mode of injection of intoxicating dose.[11]
Group 1	24 hrs.	++++	+	++	Intravenous.
Group 2	48 hrs.	++	+	+	Intravenous.
Group 3	72 hrs.	+	0	++++	Intravenous.

These experiments give somewhat irregular results, but in general they indicate that sensitization is dependent on the egg-albumin content of the blood rather than on a retention of egg-albumin in the tissues of the organs. The irregularities in both series are difficult to explain, but such irregularities are not infrequent in sensitization experiments.

To settle the question of whether the egg-white was merely present in the blood or whether it was fixed by the cells of the organ, the experiments summarized in series V were undertaken. In these the sensitizing power of the blood of a rabbit was compared with the same power of extracts of the washed and unwashed kidneys.

The method was as follows. Under the ether anesthesia a rabbit was bled from the carotid artery to secure five cubic centimeters of defibrinated blood. The abdomen was then opened, and the vessels of one kidney were ligated, and the other kidney was washed out with about 1,000 cubic centimeters of physiological salt solution through a cannula in the aorta. Guinea pigs were sensitized with the blood from the carotid and with extracts of the two kidneys.

SERIES V.

Egg-White. Comparison of Sensitizing Power of Blood and of Extracts of Washed and of Unwashed Kidney.

Guinea pigs.	Time.	Blood.	Unwashed kidney.	Washed kidney.	Mode of injection.
Group 1	24 hrs.	++++	+++	+	Intravenous.
Group 2	48 hrs.	+++	+++	+	Intravenous.
Group 3	72 hrs.	++	++	0	Intravenous.
Group 4	96 hrs.	0	++	0	Intravenous.

[11] In this and in most of the experiments that follow, the intraperitoneal administration of the intoxicating dose was abandoned on account of the more definite results obtained by the intravenous method.

These results are conclusive. In the normal animal the power of sensitization depends on the egg-albumin content of the blood and not upon a peculiar fixation of the foreign protein in the kidney. It does not necessarily follow that the same is true for all protein substances, but these observations tend, nevertheless, to support the view that injury to the kidney by a particular substance is coincident with the period of elimination of that substance by the kidney, and not to a fixation in the parenchymatous cells.

Another problem, however, remained. Is a foreign protein eliminated as readily by the diseased kidney as it is by the normal kidney? This problem could perhaps have been more exactly solved if the experiments had been made on animals with a chronic renal lesion, but the difficulty of producing the latter with any degree of constancy compelled the use of animals with acute lesions due either to uranium nitrate or to potassium chromate.

The results obtained with three different groups of rabbits suffering from nephritis due to uranium nitrate are combined in the table that follows.

SERIES VI.

The Sensitizing Power of Kidney Extracts from Animals that Received Egg-Albumin After Developing a Uranium Nephritis.

Each rabbit received 0.0075 or 0.015 gm. of uranium nitrate subcutaneously. Twenty-four hours later, when coagulable protein was demonstrable in the urine, each received in the ear vein 10 c.c. of egg-albumin diluted with 30 c.c. of salt solution. The other procedures were the same as in the earlier experiments.

No. of animal.	Time.	Mode of injection.	Result.
Guinea pig 1	24 hrs.	Intraperitoneal.	+++
Guinea pig 2	24 hrs.	Intravenous.	++++
Guinea pig 3	48 hrs.	Intravenous.	0
Guinea pig 4	48 hrs.	Intraperitoneal.	0
Guinea pig 5	48 hrs.	Intravenous.	0
Guinea pig 6	48 hrs.	Intraperitoneal.	0
Guinea pig 7	48 hrs.	Intravenous.	++++
Guinea pig 8	72 hrs.	Intraperitoneal.	++
Guinea pig 9	72 hrs.	Intravenous.	++++
Guinea pig 10	72 hrs.	Intravenous.	++++
Guinea pig 11	72 hrs.	Intravenous.	++++
Guinea pig 12	72 hrs.	Intraperitoneal.	+
Guinea pig 13	72 hrs.	Intravenous.	++
Guinea pig 14	96 hrs.	Intraperitoneal.	0
Guinea pig 15	96 hrs.	Intravenous.	0
Guinea pig 16	96 hrs.	Intravenous.	0

These results are in general similar to those obtained in normal animals, but there is here a greater tendency to sensitization by extracts made as late as the third day (compare series I, III, and V). An acute nephritis due to uranium nitrate apparently lengthens slightly the period during which the kidney (or blood?) may retain a foreign protein. The difference is, however, slight. No explanation is at hand for the curious negative results in four of the five experiments with forty-eight hour extracts.

In another experiment, potassium chromate was used as the renal irritant, and the guinea pigs were sensitized as in series V with blood and with the extracts of washed and unwashed kidneys.

SERIES VII.

Comparison of Sensitizing Power of Blood and of Extracts of Washed and Unwashed Kidneys from Animals that Received Egg-Albumin After Developing a Chromate Nephritis.

Each rabbit was injected subcutaneously with 0.3 gm. of potassium chromate, and on the following day, after coagulable protein had been demonstrated in the urine, received in the ear vein 10 c.c. of egg-white diluted with 30 c.c. of salt solution. Blood was drawn and the extracts of washed and unwashed kidney were made after 1, 2, 3, 4, and 5 days. All intoxicating doses were given intravenously.

Guinea pigs.	Time.	Blood.	Unwashed kidney.	Washed kidney.
Group 1	24 hrs.	++++	++++	++++
Group 2	48 hrs.	+++	++++	++
Group 3	72 hrs.	++	++	+
Group 4	96 hrs.	++++	+++	+
Group 5	108 hrs.	0	++++	0

Here we have definite evidence of a persistence of the egg-albumin in the blood, as is shown by the sensitizing power of the blood and of the extracts of unwashed kidney on the fourth day, a period greater by twenty-four to forty-eight hours (series I, III, and V) than that in the normal animal. The discordant result with the unwashed kidney extract of the fifth day cannot be explained, but it is in keeping with the irregular results seen in other series (series III, IV, and VI). These irregularities are due in all probability to the variations in the susceptibility of the guinea pig to sensitization, but may possibly be due, also, to variations in the power of the rabbits to eliminate a foreign protein.

This variation in the results is the one weak point of this investigation. However, the results, in general, agree, and a comparison of normal animals with those having nephritis shows that in nephritis there is a delay in elimination of about twenty-four to forty-eight hours, and this delay is too constant to be due to experimental errors in the method employed.

DISCUSSION.

This investigation is based on the assumptions that parenterally introduced protein is removed in part by the kidney unaltered, is in part destroyed (digested?) within the tissues, and that the power of the blood or of an extract of the kidney to sensitize against a certain protein is evidence of the presence of that protein in the blood or kidney used for sensitization. With this goes the assumption that until the protein is completely digested or removed, it is being eliminated by the kidney, and that therefore the period of its persistence in the kidney can be determined by sensitization tests. That the egg-albumin is actually eliminated by the kidney and occurs in the urine in a form and in sufficient amount to sensitize guinea pigs is shown by the following experiment.

Ten c.c. of egg-albumin diluted with 0.85 per cent. salt solution were injected into the ear vein of a rabbit and six hours later the urine of the animal was obtained by catheterization. Each of three guinea pigs received 2 c.c. of this urine intraperitoneally. After twenty-four hours the rabbit was again catheterized and the same amount of urine was injected into each of a group of three guinea pigs. Nine days later one guinea pig of each group received in the jugular vein 1 c.c. of egg-albumin diluted with salt solution. The disturbance which resulted was of slight severity and of doubtful significance. At the end of three weeks, however, a similar treatment of the four remaining guinea pigs resulted in typical symptoms and the death of all the animals. On autopsy they showed the characteristic lungs of anaphylaxis. There can be no doubt, therefore, that in the rabbit the intravenous introduction of egg-albumin is followed by its elimination by the kidney in a form capable of causing sensitization. No attempt has been made to determine how long the sensitizing power of the urine persists.

From other experiments, however, there is considerable evidence that the larger part of the injected egg-albumin is eliminated with the urine during the first forty-eight hours after injection. Thus, in the urine of the first day, and to some extent in that of the

second day, there appears on heating either a solid clot or a heavy, coarse, flocculent precipitate which settles rapidly and is quite different from the fine, granular, slowly settling precipitate of the serum proteins of nephritis. After forty-eight hours little or no precipitate is obtained. The possibility that the coagulable protein is in part composed of native serum albumin cannot be denied, but the prompt disappearance of coagulable protein after forty-eight hours is not characteristic of any known acute experimental nephritis giving so large a precipitate in the first twenty-four hours. As normal urine has the power through its non-coagulable protein content, according to Wells and others, to sensitize guinea pigs to serum proteins, the anaphylaxis reaction for such proteins cannot be used as a differential control and has, therefore, not been attempted in this investigation.

It has been assumed, therefore, that most of the coagulable nitrogen of the urine after intravenous injection of egg-albumin is indeed egg-albumin, and in order to determine the rate of its elimination, for comparison with the sensitization tests, determinations of the total nitrogen of the egg-white mixture injected, and of the coagulable protein of the urine, have been made. The following experiment is illustrative.

On April 26, 1912, a rabbit received in the ear vein 40 c.c. of a 1:3 mixture of egg-albumin. The nitrogen of 40 c.c. of this mixture, as estimated by the Kjeldahl method, was 0.191 gm.

Nitrogen in coagulable protein of urine of the first period of 20 hrs... 0.096 gm.
Nitrogen in coagulable protein of urine of the second period of 28 hrs. 0.0441 gm.
Nitrogen in coagulable protein of urine of the third period of 24 hrs.. 0.0054 gm.
Nitrogen in coagulable protein of urine of the fourth period of 24 hrs. 0.005 gm.

Other observations follow:

I. Total nitrogen in 40 c.c. of egg-white dilution (10 c.c. egg-white). 0.178 gm.
Nitrogen in coagulable protein of urine of the first 48 hrs..... 0.094 gm.
Nitrogen in coagulable protein of urine of the second 48 hrs..... 0.017 gm.

II. Total nitrogen in 40 c.c. of egg-white dilution..... 0.177 gm.
Nitrogen in coagulable protein of urine of the first 48 hrs..... 0.106 gm.
Nitrogen in coagulable protein of urine of the second 48 hrs..... 0.004 gm.

It is impossible to say that the coagulable protein of the urine does not contain serum protein, but its prompt decrease after forty-eight hours favors the supposition that it is mainly egg-albumin.

If this interpretation is correct, considerably more than half is eliminated within forty-eight hours and only very small amounts during the third and fourth days; the remainder is, presumably, digested or otherwise changed in the animal tissues. Thus the evidence concerning the elimination of egg-white obtained in this way supports the results of the sensitization experiments, for the stronger anaphylaxis was always obtained with the first and second day extracts, and the weaker reactions and the failures with extracts representing later periods.

In connection with the question of possible sources of error in these experiments another question arises—that of the effect of foreign protein on the normal kidney. If egg-albumin is toxic to the cells of the kidney, the experiments here described as demonstrating elimination in normal animals are really experiments on animals with injured kidneys. Vaughan, it may be remembered, believes that the kidneys of animals receiving egg-white are seriously injured. In the present investigation histological examination of the kidney was made only when an animal died before the regular period set for making extracts. In several instances a definite exudate about the glomerular tuft, markedly distending Bowman's capsule, has been seen. Whether this represents an exudate of serum albumin or of the foreign egg-albumin coagulated by the fixing agent we have found no means of determining. The prompt disappearance (after forty-eight to seventy-two hours) of coagulable protein from the urine has, however, seemed to indicate that the kidney is not seriously injured by a single injection and that the fine granular precipitate in the glomerular spaces is, therefore, in all probability egg-albumin.

SUMMARY.

Extracts of the kidneys of normal rabbits prepared one, two, three, and four days after the intravenous injection of egg-albumin and horse serum have the power to sensitize guinea pigs to a second injection of these proteins. The sensitization by first and second day extracts was constant and intense, that by the third day extracts was less marked and sometimes was not evident, and that by the fourth day extracts was only occasional, and when present was always weak.

Comparative studies of the power of the blood, liver, and kidney to sensitize, indicate that this sensitization depends on the content of foreign protein in the circulating blood and not upon its accumulation or fixation in the tissues of an organ. This opinion is supported by other experiments in which the sensitizing power of the blood and of the extracts of unwashed kidneys was compared with the sensitizing power of extracts of washed kidney.

The weak sensitizing power of washed kidney extract is taken as evidence that foreign proteins of the kinds used are not held in the tissues of the kidney, and if these results may be applied to nephrotoxic proteins, it follows that nephritis is not due to selective and persisting fixation of a protein by the renal cells, but is due to the action of such protein merely during the process of its elimination.

In experimental acute nephritis of the type due to uranium nitrate, the power of sensitization to egg-albumin is prolonged for twenty-four hours, and in the chromate type for forty-eight hours, thus indicating that in nephritis, of the acute type at least, the elimination of a foreign protein is delayed.

Attempts to study by the same methods the elimination of vegetable and bacterial proteins have failed.

THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

I. Reactions to Hemolytic Serum at Various Intervals after Splenectomy.*

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This investigation was originally undertaken for the purpose of determining the relation which might exist between the tumefaction of the spleen and the jaundice so characteristic of acute blood destruction by hemolytic poisons. As the work progressed, however, it became evident that a problem of much wider scope was before us and that it was necessary to study the general relation of the spleen to blood destruction and its sequelæ.

In the early experiments in which was studied the effect of hemolytic serum in the absence of the spleen, very definite and constant results in connection with the question of jaundice were obtained, but, as the time after splenectomy increased, other factors entered to such a degree that it became necessary to attack the problem from many sides. In order, therefore, to prepare the way for a rational consideration of the many aspects of the problem, this communication will be devoted to a general statement (1) of the relation between the hemoglobinuria and the jaundice occurring in normal animals and in splenectomized animals receiving hemolytic serum, and (2) of certain changes in the blood and urine apparently dependent on splenectomy. The explanation of these will be discussed in subsequent papers.

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METHODS.

The blood-destroying agent employed in the present series of experiments was hemolytic immune serum prepared by injecting rabbits with defibrinated blood of the dog. Five injections of five to ten cubic centimeters at intervals of five to seven days gave, as a rule, a very active hemolytic serum.

The operation of splenectomy was done aseptically under ether anesthesia. Female dogs were used almost exclusively in order that the tests for hemoglobin and bile might be made on urine obtained by catheterization. This we have found to be a most important precaution, for not infrequently the cage urine of apparently healthy dogs contains bile, and admixture of biliary pigments cannot be avoided if diarrhea or vomiting is present. All injections of hemolytic serum were made under ether anesthesia into a small superficial vein of the leg or into the jugular vein. Each experiment on a splenectomized animal was controlled by the injection of a normal animal with the same serum. When the animals were of approximately the same weight they received the same amounts of serum; but when the weight varied more than half a kilo, they received, with a few exceptions, corresponding doses per kilo of body weight.

The urine of all animals was examined for coagulable protein, hemoglobin, and bile pigments. In regard to the latter it may be said that little attention has been paid to the staining of the sclera or to the general staining of the tissue as evidence of jaundice. The appearance of bile pigments in the urine has been taken as the surest indication of jaundice, and the simple tests (Gmelin, Rosenbach) have, as a rule, been used, though in doubtful cases the more delicate tests were tried, and in several series tests for bile salts were made as a matter of routine.

THE FAILURE OF JAUNDICE DURING SHORT PERIODS AFTER SPLENECTOMY.

The effect of a powerful hemolytic serum upon the recently splenectomized animal is not widely different from that upon the normal animal. In both the blood is laked freely and death results in a few hours, thus indicating no increase in resistance in the splenectomized animal. This is shown in experiment I.

EXPERIMENT I.

A normal dog weighing 8,220 gm., and a dog splenectomized three days before, weighing 9,025 gm., received hemolytic serum intravenously in proportions of 0.5 c.c. per kilo of body weight. Both vomited shortly after receiving the serum, became progressively weaker, and died after a few hours. No urine could be obtained by catheterization and none was found in the bladder of either after death.

At autopsy both animals presented certain lesions in common, e. g., fluid in the peritoneal cavity, congestion of the liver, and edema of the lymph nodes, kidneys, and gall-bladder. The control had the greatly distended, soft, black, spodogenous spleen that is characteristic of acute blood destruction. In the splenectomized animal there were petechial hemorrhages of the serous membranes and of the mucosa of the intestine; these were not seen in the control dog. The heart and blood-vessels contained loose clots, and the serum of each animal showed, after centrifugalization, deep staining with hemoglobin.

Histological examination of tissues from the control dog showed intense congestion of the liver with general necrosis and hyaline thrombi in the blood-vessels. The sinuses of the spleen were distended by fused hyaline masses of red blood cells and the pulp was more or less necrotic. The vessels of the kidney contained hyaline masses, and the epithelium of the tubules showed granular degeneration. The lymph nodes were edematous and in them were small areas of necrosis. The liver, kidney, and lymph nodes of the splenectomized dog presented a similar histological picture.

The extreme opposite of the above experiment is shown in experiment II, in which a weak serum was used.

EXPERIMENT II.

Effect of Hemolytic Serum Administered Three Days after Splenectomy, with Control.

Date.	Dog 11. Weight 10,000 gm.	Dog 13. Weight 8,990 gm.
Dec. 11, 1911.	Splenectomy.	Control.
Dec. 14, 1911.	Urine normal; 2.5 c.c. hemolytic serum in vein.	Urine normal; 2.5 c.c. hemolytic serum in vein.
Dec. 15, 1911.	Urine normal.	Bile test positive; no hemoglobin, no albumin.
Dec. 16, 1911.	Urine normal; 4.5 c.c. of same serum in vein.	Bile test positive; no hemoglobin, no albumin; 4.5 c.c. of same serum in vein.
Dec. 17, 1911.	Trace of albumin; no bile, no hemoglobin.	Bile test positive; albumin present, no hemoglobin.
Dec. 18, 1911.	Trace of albumin; no bile, no hemoglobin.	Bile test positive; albumin present, no hemoglobin.
Dec. 19, 1911.	Trace of albumin; no bile, no hemoglobin.	Bile test positive; albumin present, no hemoglobin.
	Ligation of common bile duct under ether anesthesia.	Died, under ether, during operation to remove spleen.
Dec. 20, 1911	Bile pigments in urine.	
Dec. 21–22, 1911.	Bile pigments in urine increasing.	
Dec. 23, 1911.	Bile pigments in urine increasing. Killed by chloroform.	

In this experiment the hemolytic serum was not powerful enough to cause a destruction of blood of sufficient grade to produce hemoglobinuria, although it did cause in the control animal enough destruction to produce jaundice; on the other hand, the splenectomized animal was free from jaundice.

Three possible explanations of this difference in results are at hand: first, that the absence of the spleen has an influence on the elimination of bile; second, that splenectomy influences in some way the series of changes by which blood pigments are converted into bile pigments; and third, that owing to the difference in the weight of the animals, the splenectomized animal received a relatively smaller dose, too small, perhaps, to produce a degree of blood destruction capable of causing bile pigments to appear in the urine.

The first of these possible explanations may be dismissed at once in view of the appearance of bile pigments in the urine of the splenectomized dog within twenty-four hours after ligation of the common duct. The second is the main problem discussed in this and succeeding papers. The third explanation may be dismissed in view of the result in experiment III in which a stronger serum was used and the amount injected was adjusted to the weight of the animals employed.

EXPERIMENT III.

Effect of Hemolytic Serum Six Days after Splenectomy, with Control.

Date.	Dog 3. Weight 6,400 gm.	Dog 7. Weight 10,580 gm.
Dec. 14, 1911.	Splenectomy.	Control.
Dec. 20, 1911.	Urine normal.	Urine normal.
	0.5 c.c. serum per kilo.	0.5 c.c. serum per kilo.
Dec. 21, 1911.	Hemoglobinuria; no bile pigment.	Hemoglobinuria; marked jaundice.
Dec. 22, 1911.	Hemoglobinuria; no bile pigment.	Hemoglobinuria; marked jaundice.
Dec. 23, 1911.	Hemoglobinuria; no bile pigment.	No hemoglobinuria; marked jaundice.
Dec. 24, 1911.	Trace of hemoglobinuria; no bile pigment.	No hemoglobinuria; marked jaundice.
Dec. 25, 1911.	No hemoglobinuria; no bile pigment.	No hemoglobinuria; marked jaundice.
Dec. 26–27, 1911.	Faint trace of bile pigment.	Much bile pigment in urine.
Dec. 28, 1911.	No bile pigment.	Much bile pigment in urine.
Dec. 29, 1911.	No bile pigment.	Much bile pigment in urine.

At autopsy, dog 3 showed no bile staining of the tissues. All the organs appeared normal and the lymph nodes were not enlarged. The gall-bladder was

distended by a large amount of dark green bile. Dog 7 presented the usual large, swollen, black, spodogenous spleen; the kidneys, on section, were brownish in color, the lymph nodes were swollen, and the gall-bladder was filled with dark brown bile. Bile staining of the tissue could not be demonstrated.

In this experiment the injection of serum caused well marked hemoglobinuria in both dogs, accompanied in the normal dog by a persisting excretion of large amounts of bile pigments in the urine, but without evidence of jaundice in the splenectomized dog. On only two days did the splenectomized dog show the faintest trace of bile in the urine, and on these days the reaction was so faint as to be classed as doubtful.

EXPERIMENT IV.

Effect of Serum Fifteen Days after Splenectomy.

Date.	Dog 4. Weight 9,100 gm.	Dog 6. Weight 8,800 gm.
Nov. 14, 1911.	Splenectomy.	Control.
Nov. 25–28, 1911.	Urine normal.	Urine normal.
Nov. 29, 1911.	0.5 c.c. serum per kilo.	0.5 c.c. serum per kilo.
Nov. 30, 1911.	Marked hemoglobinuria; no bile pigment.	Marked hemoglobinuria; bile pigment abundant.
Dec. 1, 1911.	No hemoglobinuria; no bile pigment.	Trace of hemoglobinuria; much bile pigment.
Dec. 2, 1911.	No hemoglobinuria; no bile pigment.	No hemoglobinuria; much bile pigment.
	Died.	Chloroformed.

At autopsy both dogs showed focal necroses of the liver, brownish discoloration of the kidneys, and swollen edematous lymph nodes. The spleen of the control dog presented the usual spodogenous tumefaction. In this animal the lighter tissues were stained a faint yellow, which was not the case in the splenectomized animal. In each, the gall-bladder was distended and contained thick, blackish brown bile.

These experiments have one uniform result; in the splenectomized animal, jaundice failed to follow hemoglobinuria. Moreover, in the splenectomized dog, jaundice failed to follow those grades of blood destruction which, as in experiment II, caused jaundice in the normal animal, but were too slight to cause hemoglobinuria.

The literature of jaundice contains, so far as we are aware, only one series of observations on the effect of hemolytic serum in splenectomized animals, and from these observations erroneous con-

clusions have been drawn. Joannovics,[1] in his studies of the hemolytic jaundice caused by toluylendiamin, found, as had Banti[2] and Vast,[3] that splenectomized dogs require larger doses of the drug to cause jaundice than do normal animals and he agreed, therefore, with the conclusion of Vast, that for the destruction of the blood cells by toluylendiamin, the intervention of the spleen is necessary. In the course of his work, the effect of hemolytic immune serum was tried on three splenectomized dogs. The spleen of two had been removed eight days before the injection of the serum; the time of removal in the third is not stated. Two of the animals died within a few hours and one was killed on the ninth day. In none did hemoglobinuria or jaundice appear, and Joannovics concludes that the presence of the spleen is essential for the production of the hemoglobinuria due to hemolytic serum.

That this is not true is shown by our experiments (III and IV) in which hemoglobinuria was well marked. The different result in Joannovics' experiments was probably due to the method of injection, the subcutaneous, which does not yield the same degree of blood destruction as does the intravenous injection. It is of interest, however, in view of our results, that in his experiment (VII) in which a dog received, eight days after splenectomy, a relatively large dose of serum (nearly one cubic centimeter per kilo), no jaundice occurred during a period of nine days.

Experiments having for their object an explanation of the failure of hemolytic serum to cause jaundice during short periods after splenectomy are presented elsewhere. It may be stated here, however, that the phenomenon is not due to an influence of the spleen upon the transformation of hemoglobin,[4] but is apparently dependent on the changes in the blood consequent upon splenectomy.[5]

[1] Joannovics, G., Experimentelle Untersuchungen über Ikterus, *Ztschr. f. Heilk.*, 1904, xxv, 25.

[2] Banti, cited by Joannovics, *loc. cit.*

[3] Vast, cited by Joannovics, *loc. cit.*

[4] Pearce, R. M., Austin, J. H., and Eisenbrey, A. B., II. The Relation of Hemoglobinemia to Hemoglobinuria and Jaundice in Normal and Splenectomized Animals, *Jour. Exper. Med.*, 1912, xvi, 375.

[5] Pearce, R. M., Austin, J. H., and Musser, J. H., Jr., III. The Changes in the Blood Following Splenectomy and Their Relation to the Production of Hemolytic Jaundice, *Jour. Exper. Med.*, 1912, xvi, in press.

Moreover, it may be added that if splenectomy is not accompanied by anemia, on the administration of hemolytic serum jaundice occurs in the splenectomized as in the normal animal.

THE INCREASED RESISTANCE OF RED CELLS TO HEMOLYSIS AFTER SPLENECTOMY.

As the time after splenectomy lengthened, a new factor came into consideration. This was the increased resistance of the red blood cells to the action of a hemolytic serum, observed first after twenty-seven days.

EXPERIMENT V.

Increased Resistance to Hemolysis Twenty-seven Days after Splenectomy.

Date.	Dog 1. Weight 7,940 gm.	Dog 8. Weight 6,880 gm.
Nov. 10, 1911.	Splenectomy.	Control.
Dec. 5, 1911.	Urine normal.	Urine normal.
Dec. 6, 1911.	Urine normal.	Urine normal.
	0.5 c.c. serum per kilo into vein.	0.5 c.c. serum per kilo into vein.
Dec. 7, 1911.	No hemoglobin or bile pigment in urine; large amount of coagulable protein.	Severe hemoglobinuria; much bile pigment in urine.
	A second injection of 5 c.c. serum into vein.	No second injection.
Dec. 8, 1911.	Well marked hemoglobinuria; faint trace of bile pigments.	Slight hemoglobinuria; moderate bile pigment.
Dec. 9, 1911.	No hemoglobinuria; trace of bile. Chloroformed.	No hemoglobinuria; trace of bile. Chloroformed.

At autopsy dog 1 showed numerous focal necroses in the liver, congested and hemorrhagic lymph nodes, and brownish discoloration of the kidneys. The gall-bladder contained a very dark bile.

Dog 8 had the typical, firm, swollen, bluish red, spodogenous spleen and also focal necroses of the liver, congested edematous lymph nodes, and brownish discoloration of the kidneys. The gall-bladder contained a black bile thicker than that of dog 1.

In this experiment the increased resistance of the splenectomized dog's corpuscles was very clearly shown. Not only did this animal receive the same relative dose per weight, but actually 0.5 of a cubic centimeter more than did the control animal. Moreover, a second injection of five cubic centimeters caused, in so far as hemoglobinuria may be taken as an index, a less severe grade of blood destruction than in the control animal. In contrast to the previous

experiments it will be noted that the urine of the splenectomized animal contained bile pigments, but not as great an amount as the urine of the control animal.

Another experiment of the same character follows.

EXPERIMENT VI.

Increased Resistance to Hemolysis Sixty-five Days after Splenectomy.

Date.	Dog 10. Weight 9,720 gm.	Dog 22. Weight 6,710 gm.
Dec. 9, 1911.	Splenectomy.	Control.
Feb. 12, 1912.	Urine normal.	Urine normal.
Feb. 13, 1912.	0.25 c.c. serum per kilo into vein.	0.25 c.c. serum per kilo into vein.
Feb. 14, 1912.	Urine free from hemoglobin and bile pigment.	No hemoglobinuria; bile pigments present.
	1 c.c. same serum per kilo into vein.	1 c.c. per kilo of same serum into vein.
Feb. 15, 1912.	No hemoglobinuria; no bile pigment.	Marked hemoglobinuria; much bile pigment.
Feb. 16, 1912.	No hemoglobinuria; no bile pigment.	No hemoglobinuria; much bile pigment.
	2 c.c. per kilo of another serum into vein.	No third injection. Spleen excised.
Feb. 17, 1912.	Hemoglobinuria; faint trace of bile pigment.	Well marked bile reaction.
Feb. 18, 1912.	No hemoglobinuria; faint trace of bile (?).	Well marked bile reaction.
Feb. 19, 1912.	No hemoglobinuria; faint trace of bile (?).	Well marked bile reaction.
	Common bile duct ligated.	
Feb. 20–21, 1912.	Large amount of bile pigment in the urine.	Well marked bile reaction.
	Chloroformed.	Chloroformed.

At autopsy dog 10 showed general jaundice and fatty changes in the liver, kidneys, and heart. In dog 22, fatty changes were evident, but were not so marked, and no definite bile staining of the tissues could be seen.

This experiment demonstrates most conclusively the increased resistance of the red blood cells of a splenectomized animal to hemolysis. A small dose (0.25 of a cubic centimeter per kilo) of a weak serum caused in the sound dog sufficient destruction of red cells to lead to the appearance of bile pigments in the urine; the splenectomized animal was not affected. A larger dose (one cubic centimeter per kilo) of the same serum produced hemoglobinuria in the control, but no change in the splenectomized animal. Finally, a third injection of two cubic centimeters of serum per kilo, an unusually large dose, was necessary before hemoglobinuria was produced in the splenectomized animal.

This evidence of increased resistance in the splenectomized animal was supported by tests *in vitro,* both as regards spontaneous hemolysis in salt solutions of different strengths, and also as regards the hemolytic power of the serum used for injection.

Incidentally, these two animals were used to demonstrate (1) that obstructive jaundice can readily be produced in the splenectomized dog (see also experiment I) by ligating the common duct, and (2) that an existing hemolytic jaundice does not immediately lessen after removal of the spleen.

The determination of the cause of this increased resistance of the red corpuscles after splenectomy is necessarily an important part of our problem. That the splenectomized animal is more resistant than the normal animal to the action of hemolytic poisons, that is, that the splenectomized dog will bear larger doses, has been noted by Banti, Vast, and Joannovics in their work with toluylendiamin, and was also observed by Pugliese and Luzzatti,[6] in their study of the action of pyrodin.

Actual demonstration by exact methods of an increased resistance of red cells after splenectomy has been shown, so far as we are aware, only by Chalier and Charlet,[7] though these investigators quote also Bottazi[8] and Viola.[9] The differences noted by Chalier and Charlet as the result of testing the fragility of the cells of oxalated blood in different strengths of salt solution are slight, but this may be due to the fact that none of their tests appear to have been made later than ten to twelve days after splenectomy. They found in three dogs that complete hemolysis occurred in solutions varying from 0.32 to 0.36 as compared to 0.32 to 0.4 in five normal dogs.

In our work we have used fresh blood obtained by puncture and have allowed it to fall into tubes as it flowed from the wound, one drop in each tube. These tubes contained salt solution decreasing

[6] Pugliese, A., and Luzzatti, T., Contributions à la physiologie de la rate, *Arch. ital. de biol.,* 1900, xxxiii, 349.

[7] Chalier, J., and Charlet, L., État de la résistance globulaire chez l'animal normal et splénectomisé, *Jour. de physiol. et de path. gén.,* 1911, xiii, 728.

[8] Bottazi, cited by Chalier, J., and Charlet, L. (with reference to Morat, J.-P., and Doyon, M., Traité de physiologie, Paris, 1904, i, 605), *loc. cit.*

[9] Viola, cited by Chalier, J., and Charlet, L. (with reference to Morat, J.-P., and Doyon, M., Traité de physiologie, Paris, 1904, i, 605), *loc. cit.*

by twentieths of 1 per cent. from 0.7 to 0.2 per cent. In normal dogs we have found that hemolysis is complete in 0.35 or 0.4 per cent. solution, with partial hemolysis, as a rule, in the next higher tube. Only once in our experiments with the blood of normal dogs has complete hemolysis occurred in 0.3 per cent. solution. In splenectomized animals, on the other hand, if a period of at least nine days has elapsed, complete hemolysis occurs in 0.25 or 0.3 per cent. solution with partial hemolysis in the next higher tube, but the hemolysis is never complete in solutions above 0.3 per cent. In a few instances in which this test has been applied both before and after splenectomy, the increase in resistance corresponded always to a difference of 0.1 per cent. in the salt solution used. No increase in resistance was noted before the ninth day, and after this period the resistance did not increase with lapse of time up to seven and a half months, which in our series is the longest period after splenectomy.

Detailed observations having for their object the determination of the cause of this increased resistance and its relation to blood destruction and regeneration will be presented in later communications.[10]

THE OCCURRENCE OF SPONTANEOUS JAUNDICE SEVERAL MONTHS AFTER SPLENECTOMY.

In view of the difficulty of causing hemolytic jaundice in the early periods, the occurrence of spontaneous jaundice several months after splenectomy was entirely unexpected. Well marked coloring of the urine by bile pigment has been seen in two animals. In one it was noted 103 days after splenectomy and in the other after seven and a half months. How soon after the removal of the spleen this spontaneous pigmentation may occur we cannot say, for in the early part of the investigation many of the dogs were watched for only a few days after operation, and were then set aside without routine examination of the urine. Thus, the occurrence of bile in the urine was, in several of the long period splenectomies, not ob-

[10] Karsner, H. T., and Pearce, R. M., IV. A Study, by the Methods of Immunology, of the Increased Resistance of the Red Blood Corpuscles after Splenectomy, *Jour. Exper. Med.*, 1912, xvi, in press.

served until the period of study preliminary to the injection of serum. For this reason we did not determine when the bile pigments first appeared in the urine of the animals mentioned above. Since our attention was called to this phenomenon, however, all animals have been carefully watched, but large amounts of bile pigments have not been observed in the urine of animals splenectomized for periods of less than three months. In two instances, however, faint traces were found after thirty days. On the other hand, we have had only four long period splenectomies to study. In two of these (three and one half and seven and one half months) spontaneous jaundice occurred and in two (nine and one half and ten months) it was not observable.

The supposition that this spontaneous jaundice might be due to a diminished resistance and to a spontaneous hemolysis of red cells is contra-indicated by the fact that the red cells of both animals presenting jaundice showed an increased resistance to lysis by both salt solution and hemolytic serum. The following experiment will illustrate this.

EXPERIMENT VII.

Spontaneous Jaundice One Hundred and Three Days after Splenectomy, with Increased Resistance of the Red Cells.

Date.	Dog 2. Weight 10,290 gm.	Dog 29. Weight 7,250 gm.
Nov. 14, 1911.	Urine normal.	
Nov. 15, 1911.	Splenectomy.	Control.
Feb. 25, 1912.	Urine deeply pigmented by bile.	Urine normal.
Feb. 26, 1912.	0.25 c.c. serum per kilo into vein.	0.25 c.c. serum per kilo into vein.
Feb. 27, 1912.	No hemoglobinuria; much bile pigment.	Marked hemoglobinuria; fairly well marked bile reaction.
Feb. 28, 1912.	No hemoglobinuria; much bile pigment.	Slight hemoglobinuria; much bile pigment.
Feb. 29, 1912.	No hemoglobinuria; much bile pigment.	Urine lost.
Mar. 1, 1912.	No hemoglobinuria; much bile pigment.	Much bile pigment; no hemoglobinuria.
	Experiment discontinued.	

At autopsy the splenectomized animal presented no disturbance of the liver or biliary passages capable of explaining the jaundice as being due to obstruction.

In a second animal a persisting and abundant pigmentation of the urine was noticed seven and a half months after splenectomy. The increased resistance of the red cells was shown by hemolysis in 0.3

per cent. salt solution, whereas the cells of the control animal were hemolyzed in 0.4 per cent. Hemoglobinuria was brought about in the control dog by an injection of 0.5 of a cubic centimeter of hemolytic serum per kilo, while in the splenectomized animal hemoglobinuria did not occur until the dose was raised to one cubic centimeter per kilo.

That the jaundice in this particular animal was not associated with an anemia is shown by the fact that the blood examination gave a red blood cell count of 6,120,000 and a hemoglobin content of 97 per cent. Our attempts to explain this phenomenon are presented in the third paper[11] of this series.

SUMMARY.

This investigation brings out three points.

1. During the first three or four weeks after splenectomy hemoglobinuria due to hemolytic serum was not followed by jaundice.

2. One month and more after splenectomy the red cells had an increased resistance to hemolysis.

3. Three and a half months after splenectomy and even later, jaundice occasionally occurred spontaneously.

Attempts to explain these three phenomena have been based on the following possibilities: (1) that some function of the spleen is essential for the degradation of hemoglobin or for the elaboration of its derivatives; (2) that there is some interrelation between the spleen and the blood-forming organs or between the spleen and the blood-disintegrating mechanism, or perhaps both interrelations exist; (3) that there is a disturbance of the hemolyzing or bile-forming function of the liver dependent on the blood changes following splenectomy.

This multiplicity of problems and likewise of possible explanations has rendered the presentation of the explanatory work in one communication extremely difficult. In the interests of clearness, therefore, the various phases are dealt with in separate papers.

[11] Pearce, R. M., Austin, J. H., and Musser, J. H., Jr., *loc. cit.*

THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

II. The Relation of Hemoglobinemia to Hemoglobinuria and Jaundice in Normal and Splenectomized Animals.*

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This publication is the second of a series on the general subject of the influence of the spleen on blood degeneration and regeneration and the production of hemolytic jaundice, and has for its object the establishment of a satisfactory theory to explain, on the one hand, the quantitative relations of hemoglobinuria and jaundice, and, on the other, the relative importance of the liver and kidneys in removing free hemoglobin from the circulating blood.

The main investigation, to which the present study bears only an incidental relation, was undertaken for the purpose of determining the relation, if any, which may exist between the peculiar (spodogenous) enlargement of the spleen and the jaundice so characteristic of acute destruction of the blood by hemolytic agents.

In early experiments in which hemolytic serum was administered to dogs shortly after splenectomy, jaundice not infrequently failed to appear. The first, and perhaps natural conclusion was that the spleen is in some way concerned in the preparation of hemoglobin for action by the liver cells, but after repeated attempts had been made to explain the phenomenon it became evident that the problem was not easy of solution and that fundamental observations on the fate of free hemoglobin in the circulating blood were necessary as

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a basis for further investigation. The study of the fate of known quantities of hemoglobin injected into normal and splenectomized animals was therefore undertaken, and it is the results of this study which are here presented. The points studied were: (1) the degree of hemoglobinemia necessary in order to recognize free hemoglobin in the serum; (2) the degree of hemoglobinemia necessary for the escape of hemoglobin through the kidneys; (3) the percentage of hemoglobin eliminated by the kidneys; (4) the degree of retention of hemoglobin necessary to cause jaundice; (5) the influence of the absence of the spleen on the elimination or retention of hemoglobin.

METHODS.

Defibrinated dog blood was hemolyzed with distilled water, sodium chlorid was added to render the solution isotonic with dog blood, the diluted blood was centrifugalized, and the hemoglobin content was determined by Fleischl's method. Definite amounts of the hemoglobin solution, always freshly prepared, were allowed to flow from a burette into a small branch of the femoral vein. The first appearance of hemoglobin in the urine was determined by a catheter in the bladder only or by a second catheter in one ureter. In order to aid the flow of urine each dog received 300 cubic centimeters of water by stomach tube. From time to time the skin was punctured and the blood was drawn into capillary tubes to determine how early free hemoglobin appeared in the serum.

The elimination of hemoglobin in the urine was estimated by rendering the urine acid with hydrochloric acid to about N/10 and comparing this solution of acid hematin, suitably diluted, with a 1 per cent. solution made according to the Sahli method from blood containing 100 per cent. of hemoglobin by the Fleischl scale. A Duboscq colorimeter was used for making the comparison.

Quantities of hemoglobin are designated throughout the paper in grams calculated on the assumption, for the sake of convenience, that blood giving a reading of 100 per cent. by the Fleischl scale contains 14 per cent. of hemoglobin. This figure is, of course, only approximately correct, but as relative quantities are of importance in our work, an approximate determination of the absolute quantities of hemoglobin is sufficient.

It is of the utmost importance that all urine containing hemoglobin be placed promptly in the refrigerator until the colorimetric estimation is made. If such urine is allowed to stand a few hours at room temperature a green precipitate of a hemoglobin derivative is formed which, when treated with sodium chlorid and glacial acetic acid, gives the hemin test.

The experiments were made almost exclusively on female dogs, in order that urine obtained by catheterization might be available for the bile tests. This has been found to be most important on account of the small amounts of bile pigments occurring in the urine under the condition of these experiments. In all experiments ether anesthesia was employed.

The earlier experiments, which are given below, were made with the object of determining approximately (a) how much free hemoglobin is necessary to stain the serum, (b) how much must be introduced to cause its appearance in the urine, and (c) to gain some idea of the time relations.

Experiment I.—Normal dog, female; weight 8,360 gm. Ether anesthesia; cannula in left femoral vein; catheter in bladder; 300 c.c. of water by stomach tube.

Feb. 21, 1912. 3.20 P. M. Urine normal.

3.22 P. M. Blood serum clear.

3.25 P. M. 10 c.c. of hemolyzed blood injected (equal to 0.32 gm. of hemoglobin).

3.30 P. M. Blood serum clear.

3.32 P. M. 10 c.c. of hemolyzed blood injected.

3.37 P. M. Blood serum clear.

3.39 P. M. 10 c.c. of hemolyzed blood injected.

3.40 P. M. Much albumin in urine.

3.44 P. M. Blood serum faintly tinged with hemoglobin.

3.51, 3.58, 4.05, 4.12, 4.19, 4.26 P. M. Blood serum deeply stained with hemoglobin.

3.46, 3.53, 4.00, 4.07, 4.14, and 4.21 P. M. 10 c.c. of hemolyzed blood injected.

3.55, 4.15, 4.21 P. M. Much albumin in urine.

4.22 P. M. Hemoglobin in urine.

5.23 P. M. Hemoglobin in urine decreasing in amount. Total elimination of hemoglobin = 0.18 gm.

8.00 P. M. Urine free of hemoglobin.

Feb. 22–29, 1912. Urine free of hemoglobin; no bile pigment; slight trace of albumin on 22d and 23d; none after this.

Summary.—The total amount of hemoglobin injected was 2.91 gm. (0.35 gm. per kilo); the total elimination by the kidneys was 0.18 gm. The hemoglobin

retained was 2.91 — 0.18 = 2.73 gm., or 0.33 gm. per kilo. Hemoglobin was demonstrated in the blood serum after a lapse of nineteen minutes when 30 c.c. of the solution (0.97 gm. of hemoglobin) had been injected, and appeared in the urine after fifty-seven minutes when 90 c.c. of the solution (2.91 gm. of hemoglobin) had been introduced. Despite the great retention of hemoglobin, bile pigments did not appear in the urine.

Experiment II.—Normal dog, female; weight 11,090 gm. Ether anesthesia; cannula in branch of right femoral vein; catheter in bladder; 300 c.c. of water by stomach tube.

Feb. 29, 1912. 3.57 P. M. Urine normal.
4.01 P. M. Blood serum clear.
4.02–4.07 P. M. 20 c.c. of hemolyzed blood injected, equal to 0.49 gm. of hemoglobin.
4.07 P. M. Blood serum clear.
4.09–4.15 P. M. 20 c.c. of hemolyzed blood injected.
4.10 P. M. Some albumin in urine.
4.14 P. M. Blood serum clear.
4.16–4.18 P. M. 20 c.c. of hemolyzed blood injected.
4.20 P. M. Much albumin in urine.
4.21 P. M. Blood serum showed a trace of hemoglobin.
4.23–4.26 P. M. 20 c.c. of hemolyzed blood injected.
4.28 P. M. Blood serum deeply colored by hemoglobin.
4.30 P. M. 10 c.c. of hemolyzed blood injected.
4.32 P. M. Large amount of albumin in urine.
4.33 P. M. Hemoglobin present in urine.
4.35 P. M. Blood serum deeply colored by hemoglobin.

March 1, 1912. The cage urine showed a trace of albumin, no bile pigment, and no hemoglobin. Urine by catheter at 10 A. M. showed no albumin.

Summary.—The total amount of hemoglobin injected was 2.19 gm. (0.20 gm. per kilo); the total elimination by the kidney was 0.05 gm. of hemoglobin. The amount retained was, therefore, 2.19–0.05 = 2.14 gm. of hemoglobin, or 0.19 gm. per kilo. Hemoglobinuria was evident after a lapse of nineteen minutes when 60 c.c. of the solution (1.46 gm. of hemoglobin) had been injected, and hemoglobin appeared in the urine after thirty-one minutes when 90 c.c. of solution (2.19 gm. of hemoglobin) had been injected. Although nearly all the hemoglobin was retained, bile pigment did not appear in the urine.

Experiment III.—Normal dog, female; weight 11,040 gm. Ether anesthesia; cannula in branch of femoral vein; catheter in bladder; 300 c.c. of water by stomach tube.

March 1, 1912. 2.30 P. M. Urine normal.
2.34 P. M. Blood serum clear.
2.35–2.38 P. M. 20 c.c. of hemolyzed blood injected (equal to 0.80 gm. of hemoglobin).
2.40 P. M. Blood serum showed faint trace of hemoglobin.
2.42–2.45 P. M. 10 c.c. of hemolyzed blood injected.
2.47 P. M. Blood serum showed faint trace of hemoglobin.
2.49–2.51 P. M. 10 c.c. of hemolyzed blood injected.
2.52 P. M. Hemoglobin present in urine.

2.54 P. M. Serum showed much hemoglobin.

March 2, 1912. 9.00 A. M. Urine obtained by catheter showed no hemoglobin, no bile pigment, and no albumin. Cage urine contained 0.15 gm. of hemoglobin.

12.00 M. Urine obtained by catheter showed no hemoglobin, no bile pigment, and no albumin.

March 3, 1912. Urine normal.

Summary.—Hemoglobin appeared in the serum five minutes after the beginning of the injection of 20 c.c. of the solution (0.80 gm. of hemoglobin), and was present in the urine after seventeen minutes, when 40 c.c. (1.60 gm. of hemoglobin) had been injected. The total amount of hemoglobin introduced was 1.6 gm. (0.14 gm. per kilo). The total amount eliminated was 0.15 gm. The amount retained was, therefore, 1.45 gm. (0.13 gm. per kilo). As in the other experiments, the retained hemoglobin did not appear in the urine as bile pigment.

In order to determine precisely the moment when the kidney begins to excrete hemoglobin, the following experiments, in which a single large injection of hemoglobin was given, were undertaken.

Experiment IV.—Normal dog, female; weight 7,100 gm. Ether anesthesia; cannula in left external jugular vein; catheter in bladder; cannula in left ureter projecting into pelvis of kidney; 300 c.c. of water by stomach tube.

March 2, 1912. 3.29–3.33 P. M. 106 c.c. of hemolyzed blood injected (= 0.34 gm. of hemoglobin.

3.39½ P. M. Hemoglobin appeared simultaneously in both vesical catheter and ureteral cannula.

Experiment V.—In another experiment of the same type a hemoglobin solution containing 0.32 gm. of hemoglobin was injected into the jugular vein between 4.04 and 4.06 P. M. Hemoglobin appeared in the ureteral cannula at 4.14½ P. M., and in the vesical catheter a few seconds later.

In these last experiments, if we take experiment IV for purposes of comparison, it is obvious that the hemoglobin concentration of the blood, determining the threshold of the kidney for hemoglobin, was reached at some time betwen 3.29 and 3.33 P. M. The detection of hemoglobin in the ureter and bladder was possible at 3.39½ P. M. It is evident from this that hemoglobin can be detected in the bladder about eight minutes after the threshold value of the kidney is reached. We may assume, therefore, that in experiments I, II, and III the threshold of the kidney for hemoglobin was reached eight minutes before hemoglobin appeared in the urine; that is, in experiment I at 4.14 P. M., at which time 2.59 grams of hemoglobin had been injected; in experiment II, at 4.25 P. M., at which time 1.95 grams of hemoglobin had been injected; and in ex-

periment III, at 2.44 P. M., at which time 1.2 grams of hemoglobin had been injected.

The question at once arises as to the reason for the apparent differences in these threshold values, experiments II and III being on the same animal. It is to be found, we believe, in the rate of injection. Thus in experiment I, the lapse of time from the beginning of the injection to the time the threshold of the kidney was reached was forty-nine minutes (3.25 to 4.14 P. M.); in experiment II, this interval was twenty-three minutes (4.02 to 4.25 P. M.), and in experiment III, it was nine minutes (2.35 to 2.44 P. M.).

It is suggested at once that even while we are injecting, the hemoglobin is being removed at a definite rate by the tissues (liver?) as well as by the kidney, and that when the threshold value is reached we have actually less hemoglobin present in the serum than the above figures would indicate. Thus, if in experiments II and III (which were on the same animal and are, therefore, comparable) we assume that from the moment the injection began, tissues other than the kidneys were removing the hemoglobin at the rate of 0.054 of a gram per minute, then in experiment II at the end of twenty-three minutes there will have been removed by the tissues 1.24 grams (23 times 0.054 of a gram) of the 1.95 grams injected, leaving 0.71 of a gram (0.06 of a gram per kilo) present in the blood serum at 4.25 P. M. when the kidney threshold was reached; and in experiment III at the end of nine minutes there will have been removed 0.49 of a gram (9 times 0.054 of a gram) of the 1.2 grams injected, leaving again 0.71 of a gram (0.06 of a gram per kilo) present in the blood serum at 2.44 P. M. when the threshold was reached. That this rate is not constant for all animals is shown in experiment I, in which the absorption took place more slowly. That the hemoglobin is nevertheless removed from the serum at about the rate of 0.05 of a gram per minute is borne out by the fact that after the injection of 2.8 to 4 grams of hemoglobin, we found the serum almost clear in about an hour or a little more. We therefore conclude that elimination of hemoglobin by the kidneys does not occur until it is present in the serum in a concentration of about 0.06 of a gram per kilo of body weight.

Concerning the amount of hemoglobin eliminated by the kidneys,

it is probable that were it possible to stop injecting at the moment the renal threshold is reached, the quantity of hemoglobin eliminated would be a mere trace that could not be estimated. To do this, however, is practically impossible since one must wait for about eight minutes in order to detect the hemoglobin in the urine after the threshold is reached; but if very small amounts of hemoglobin are injected at such long intervals the hemoglobin will be entirely removed by tissues other than the kidneys during the eight minute intervals and the threshold will never be reached.

In each of the experiments quoted it happened that one injection of hemoglobin was given after the renal threshold was reached. Thus in experiment I, at 4.21 P. M., 0.32 of a gram of hemoglobin had been injected; in experiment II, at 4.30 P. M., 0.24 of a gram of hemoglobin had been injected; and in experiment III, at 2.49 to 2.51 P. M., 0.39 of a gram of hemoglobin had been injected. The total amounts eliminated in the urine were in experiment I, 0.18 of a gram; in experiment II, 0.05 of a gram; and in experiment III, 0.15 of a gram (table I). Thus these quantities bear, as we should expect, a relation to the amount of blood injected above the threshold value; the amount eliminated being about half (or somewhat less), in excess over the threshold value. There is, of course, no relation between the total amount injected and the amount recovered in the urine, nor should we expect such a relation since the kidney is active only when a concentration has been reached which is above the threshold value for the kidney.

In none of these experiments was jaundice observed, and, as a matter of fact, we have found that in all normal animals, when injections are made slowly, jaundice is less readily produced than when the injections are made rapidly, as in the experiments to be described later.

The next problem for consideration was that concerning the degree of retention of hemoglobin necessary to cause jaundice. In order to study this question quantitatively and accurately in the shortest possible time, decreasing quantities of hemolyzed blood were injected intravenously into a series of normal dogs; in each case the percentage elimination by the kidney and occurrence or non-occurrence of bile pigments in the urine were noted.

TABLE I.

Details of Injection of Hemoglobin.

Experiment No.	Remarks.	Injection time.		Hemoglobin injected.		Hemoglobin eliminated.		Hemoglobin retained.			Choluria.
		Duration in min.	Gm. of hemoglobin per kilo per min.	Total in gm.	Amount in gm. per kilo.	Total in gm.	Per cent.	Total in gm.	Amount in gm. per kilo.	Per cent.	
I	Normal	56	0.006	2.91	0.35	0.18	6.1	2.73	0.33	93.9	None.
II	Normal	28	0.007	2.19	0.20	0.05	2.3	2.14	0.19	97.7	None.
III	Normal	16	0.009	1.60	0.14	0.15	9.6	1.45	0.13	90.4	None.
VI	Normal	13	0.045	3.36	0.58	1.09	32.5	2.27	0.39	67.5	Marked.
VII	Normal	8	0.039	2.38	0.31	0.64	26.8	1.74	0.23	73.2	Faint trace.
VIII	Normal	11	0.024	2.21	0.26	0.38	17.1	1.83	0.22	82.9	Very faint trace.
IX	Normal	4	0.072	2.03	0.29	0.73	35.9	1.30	0.18	64.1	None.
X	Before splenectomy	27	0.030	5.03	0.81	0.85	16.0	4.18	0.68	84.0	Marked.
X	After splenectomy	40	0.013	3.36	0.54	0.63	18.8	2.73	0.44	81.2	Marked.
XI	Before splenectomy	32	0.012	3.94	0.38	1.06	26.5	2.88	0.28	73.5	Faint trace.
XI	After splenectomy	8	0.046	3.92	0.37	0.66	16.8	3.26	0.31	83.2	Faint trace.
XII	Splenectomy 28 days	10	0.032	3.08	0.32	0.65	21.1	2.43	0.25	78.9	Present.
XIII	Splenectomy 9 mos.	10	0.038	6.57	0.38	1.81	27.5	4.76	0.28	72.5	Trace.
XIV	Splenectomy 61 days	5	0.056	2.09	0.28	0.15	7.2	1.94	0.26	92.9	Present.
XV	Splenectomy 60 days	7	0.039	3.46	0.27	0.64	18.4	2.82	0.22	81.6	Trace.
XVI	Splenectomy 110 days Hemolytic jaundice	10	0.025	3.92	0.25	1.12	28.6	2.80	0.18	71.4	Marked.
XVII	Splenectomy 23 days Obstructive jaundice	7	0.036	2.23	0.25	0.59	26.4	1.67	0.19	73.6	Marked.

The following experiments are illustrative.

Experiment VI.—Normal dog, female; weight 5,780 gm. Ether anesthesia; cannula in small branch of saphenous vein; catheter in bladder; 300 c.c. of water by stomach tube.

March 27, 1912. 3.20 P. M. Urine normal. Blood serum clear.

3.32–3.45 P. M. 150 c.c. of hemoglobin solution (3.36 gm. of hemoglobin) injected.

3.47 P. M. Blood serum deeply stained with hemoglobin.

3.48 P. M. Urine contained hemoglobin in small amount.

3.49 P. M. Urine deeply stained with hemoglobin.

12.00 M. Urine collected at this time contained 1.09 gm. of hemoglobin. Bile pigment present.

March 28, 1912. 8.00 A. M. The cage urine contained much bile pigment; no hemoglobin, no albumin.

10.00 A. M. Urine obtained by catheter contained bile pigment in lessened amount.

March 29, 1912. Faint trace of bile pigment in urine.

Summary.—The total amount of hemoglobin injected was 3.36 gm. (0.58 gm. per kilo). The total amount eliminated by the kidney was 1.09 gm. The amount retained was, therefore, 3.36–1.09 gm. = 2.27 gm. (0.39 gm. per kilo). Bile pigments appeared in the urine in considerable amounts.

Experiment VII.—Female dog; weight 7,700 gm. Ether anesthesia. Injections of hemoglobin solution through hypodermic needle into superficial vein of lower part of leg.

March 28, 1912. 2.00 P. M. No albumin and no bile in urine.

3.59–4.07 P. M. Injection into vein of 96.5 c.c. of hemoglobin solution (2.38 gm. of hemoglobin).

4.12 P. M. Catheterized. Hemoglobinuria was marked.

March 29, 1912. The cage urine contained much hemoglobin, a trace of bile, but no albumin. Total hemoglobin eliminated = 0.64 gm.

10.30 A. M. Urine obtained by catheter contained no hemoglobin, and no albumin, but a trace of bile.

March 30, 1912. No albumin and no bile in the urine.

Summary.—The total amount of hemoglobin injected was 2.38 gm. (0.31 gm. per kilo). The total amount eliminated by the kidney was 0.64 gm. The amount retained was, therefore, 1.74 gm. (0.23 gm. per kilo). For about twenty-four hours bile pigments occurred in traces in the urine.

Experiment VIII.—Female dog; weight 8,435 gm. Ether anesthesia. Injections through hypodermic needle into superficial vein of lower part of leg.

March 29, 1912. No bile in urine.

3.38–3.49 P. M. Injection into vein of 90 c.c. of hemoglobin solution (.21 gm. of hemoglobin).

11.50 P. M. The cage urine (31 c.c.) contained 0.38 gm. of hemoglobin. There was a light cloud of albumin and the faintest possible trace of bile.

March 30, 1912. 10.00 A. M. The cage urine contained no hemoglobin, and no bile, but a trace of albumin.

March 31, 1912. The cage urine contained no bile.

Summary.—The total amount of hemoglobin injected was 2.21 gm. (0.26 gm. per kilo). The total amount eliminated by the kidney was 0.38 gm. The amount retained was, therefore, 1.83 gm. (0.22 gm. per kilo). Bile pigments occurred in the urine for about eight hours in the faintest possible traces.

Experiment IX.—The same dog as in experiment VII; weight 7,010 gm. Ether anesthesia. Injections through hypodermic needle into superficial vein of lower part of leg.

April 2, 1912. No albumin and no bile in urine.

2.50–2.54 P. M. Injection into vein of 71.5 c.c. of hemoglobin solution (2.03 gm. of hemoglobin).

April 3, 1912. 8.00 A. M. Urine contained 0.73 gm. of hemoglobin, no bile, and no albumin.

April 4, 1912. Urine contained no bile and no albumin.

Summary.—The total amount of hemoglobin injected was 2.03 gm. (0.29 gm. per kilo). The total amount eliminated by the kidney was 0.73 gm. The amount retained was, therefore, 1.30 gm. (0.18 gm. per kilo). No bile pigments appeared in the urine.

A comparison of these four experiments on normal dogs shows that the retention of 0.39 of a gram of hemoglobin per kilo caused marked choluria; of 0.23 of a gram, slight choluria for twenty-four hours; of 0.22 of a gram, a very faint choluria for eight hours; and of 0.18 of a gram, no choluria. The threshold for jaundice by this method in the normal dog lies apparently, therefore, between about 0.18 of a gram and 0.22 of a gram of hemoglobin per kilo of body weight.

The percentage of hemoglobin eliminated by the kidney appears to be a variable quantity. Thus, in experiment VI, 32.5 per cent. of the hemoglobin injected was eliminated by the kidney; in experiment VII, 26.8 per cent.; in experiment VIII, 17.1 per cent.; in experiment IX, 35.9 per cent.

In these four experiments the hemoglobin solution was rapidly injected during a period of from four to thirteen minutes. When the solution was introduced slowly a much larger amount could apparently be cared for in the liver without the production of jaundice. Thus, if we refer again to experiment I, in which the solution was introduced at intervals throughout a period of fifty-six minutes, we find that an amount of hemoglobin was retained equal to 0.33 of a gram per kilo, without bile pigments occurring in the urine.

DISCUSSION.

These experiments seem definitely to establish the mechanism by which free hemoglobin is removed from the blood serum under normal conditions. Our conception of this mechanism is as follows. The kidney does not eliminate hemoglobin until its concentration in the blood serum reaches a certain level. This concentration we conclude from experiments I, II, and III is about that produced by the presence of 0.06 of a gram of free hemoglobin per kilo of body weight. As soon as the concentration of the hemoglobin in the serum is above this point, the hemoglobin passes through the kidneys and we have hemoglobinuria, but as soon as it falls below this amount, the hemoglobinuria ceases. However, other tissues, of which presumably the liver is the most important, appear to take up hemoglobin as soon as mere traces are present in the serum and continue to remove it from the serum whether the renal threshold is exceeded or not. Therefore, whenever the kidney is removing hemoglobin from the serum these other tissues are also removing it. The kidneys apparently remove 17 to 36 per cent., and the liver (and other tissues?) 64 to 83 per cent.

The hemoglobin which the liver removes is changed into bile pigment, which, if it is not produced in too large amounts, or if the hemoglobin is not taken to the liver too rapidly, passes out as bile pigment in the usual manner through the bile passages. On the other hand, if the hemoglobin is taken up by the liver in larger quantities, and especially if this occurs rapidly, the bile is formed faster than the bile capillaries can remove it and it is reabsorbed into the circulation and appears in the urine. Obviously if this hypothesis is correct, the larger the amount of bile pigment already present in the liver, the smaller will be the amount of hemoglobin necessary to call forth a choluria, and *vice versa*.

Whether the accumulation of bile pigment or of hemoglobin in the liver influences the rate at which the liver removes hemoglobin from the serum has not been determined with certainty, but some of the experiments (to be presented later) apparently show a greater elimination of the hemoglobin by the kidney when the liver is presumably more or less saturated, as in spontaneous and obstructive jaundice, and the natural explanation for this is, that accumulation

in the liver diminishes the rate at which the liver takes up the hemoglobin from the serum, and, consequently, more hemoglobin is left for the kidneys to remove.

The Effect of Splenectomy.—Having determined these facts concerning the elimination of hemoglobin and the production of jaundice in normal dogs, we next studied the effect of splenectomy.

In two experiments hemoglobin solution was injected into normal dogs, after which these animals were splenectomized and later the hemoglobin injections were repeated.

The experiments follow.

Experiment X.—Normal dog, female; weight 6,190 gm. Ether anesthesia; cannula in small vein of left leg; catheter in bladder; 300 c.c. of water by stomach tube.

Feb. 26, 1912. 3.25 P. M. Urine normal.

3.28–3.55 P. M. 140 c.c. of hemolyzed blood injected (5.03 gm. of hemoglobin).

4.10 P. M. Urine contained hemoglobin.

Feb. 27, 1912. 1.00 A. M. Urine contained much hemoglobin (0.80 gm. of hemoglobin). No bile pigment.

8.13 A. M. Urine obtained by catheter contained a small amount of hemoglobin (0.05 gm.). Faint but definite positive test for bile pigment.

3.00 P. M. Urine free of hemoglobin; bile test strongly positive.

4.00 P. M. Splenectomy.

Feb. 28, 1912. Moderate amount of bile pigment in urine.

Feb. 29, 1912. Trace of bile pigment in urine. Animal prepared for test as on Feb. 26. Injection into small vein of right leg.

2.30–2.42 P. M. Faint trace of bile pigment.

2.42–3.22 P. M. Slow injection of 140 c.c. of hemolyzed blood (3.36 gm. of hemoglobin).

3.05 P. M. Urine contained hemoglobin (at this time, 2.49 gm. of hemoglobin had been injected).

3.42 P. M. Urine contained much hemoglobin.

March 1, 1912. 8 A. M. The cage urine was slightly tinged with hemoglobin. The total amount eliminated was 0.63 gm. There was a faint bile reaction.

9.00 A. M. Urine obtained by catheter was free from hemoglobin, but gave a well marked bile reaction.

Summary.—Before splenectomy the amount of hemoglobin injected was 5.03 gm. (0.81 gm. per kilo), the amount eliminated by the kidneys was 0.85 gm., and the amount retained was 4.18 gm. (0.68 gm. per kilo). Bile pigment was abundant in the urine. The hemoglobin eliminated through the kidneys was 16 per cent. of the total amount injected. After splenectomy the amount of hemoglobin injected was 3.36 gm. (0.54 gm. per kilo); the amount eliminated by the kidneys was 0.63 gm., and the amount retained was 2.73 gm. (0.44 gm. per kilo). Bile pigment was abundant in the urine. The percentage of hemoglobin eliminated through the kidneys was 18.8 per cent. of the total amount injected.

In the second experiment of the same type smaller amounts of hemoglobin were used.

Experiment XI.—Normal dog, female; weight 10,500 gm. Ether anesthesia; catheter in bladder; cannula in small vein of left leg; 300 c.c. of water by stomach tube.

March 4, 1912. 4.10 P. M. Urine normal.

4.18–4.23 P. M. 50 c.c. of hemolyzed blood injected (1.97 gm. of hemoglobin).

4.45–4.50 P. M. 50 c.c. of hemolyzed blood injected (1.97 gm. of hemoglobin).

4.55 P. M. Urine contained 0.11 gm. of hemoglobin.

March 5, 1912. 8.00 A. M. The cage urine contained 0.95 gm. of hemoglobin. Bile test was faintly positive.

5 P. M. Urine obtained by catheter contained no hemoglobin and no bile.

March 6, 1912. 2.00 P. M. Urine obtained by catheter contained no hemoglobin, but had a very faint trace of bile.

4.00 P. M. Splenectomy.

March 8, 1912. The animal was prepared for test as on March 4; injection into small vein of right leg.

4.30 P. M. Urine normal.

4.32–4.40 P. M. Injection of 100 c.c. of hemolyzed blood (3.92 gm. of hemoglobin).

4.47 P. M. Urine contained hemoglobin.

March 9, 1912. Urine contained much hemoglobin and a trace of bile.

The total elimination of hemoglobin was 0.66 gm.

March 10–11, 1912. Urine showed faint trace of bile.

March 12–13, 1912. Urine free of bile.

Summary.—Before splenectomy the amount of hemoglobin injected was 3.94 gm. (0.38 gm. per kilo). The amount eliminated by the kidneys was 1.06 gm., and the amount retained was 2.88 gm. (0.28 gm. per kilo). Bile pigments appeared in the urine in traces. The percentage of hemoglobin eliminated through the kidneys was 26.5. After splenectomy the amount of hemoglobin injected was 3.92 gm. (0.37 gm. per kilo), the amount eliminated by the kidneys was 0.66 gm., and the amount retained was 3.26 gm. (0.31 gm. per kilo). Bile pigments were present in the urine in traces. The percentage of hemoglobin eliminated through the kidneys was 16.8 per cent.

In this experiment, therefore, the spleen did not have any influence on the elimination or the retention of hemoglobin, or on the retardation of the appearance of bile pigments in the urine.[1] However, as these experiments were done only a few days after splenectomy and with quantities of hemoglobin larger than the minimum

[1] Recently it has been shown by Gilbert, A., Chabrol, E., and Bénard, H. (Recherches sur la biligénie consécutive aux injections expérimentales d'hémoglobine, *Presse méd.*, 1912, xx, 113) that absence of the spleen does not influence the power of the liver to transform into bile the hemoglobin furnished in the form of laked blood.

necessary to produce jaundice in normal animals, it was thought wise to make the same test at longer periods after operation and with smaller quantities of hemoglobin.

Experiment XII.—Female dog.

March 13, 1912. Splenectomy.

April 10, 1912. Weight 9,740 gm. Ether anesthesia; needle in small vein of leg. No albumin and no bile in urine.

4.04–4.10 P. M. Injected 76 c.c. of a solution containing 2.66 gm. of hemoglobin.

April 11, 1912. 8.00 A. M. The cage urine contained 0.15 gm. of hemoglobin and also a quantity of precipitated and altered hemoglobin that could not be accurately measured; no bile pigment was present.

10.00 A. M. Urine obtained by catheter contained no hemoglobin and no bile.

4.00–4.10 P. M. Injected 122 c.c. of a solution containing 3.08 gm. of hemoglobin.

April 12, 1912. 8.00 A. M. The cage urine contained 0.65 gm. of hemoglobin, a trace of bile, and a light cloud of albumin.

9.30 A. M. Urine obtained by catheter showed moderate bile test; no albumin.

April 13, 1912. No bile in urine.

Summary.—At the first injection, 2.66 gm. of hemoglobin (0.27 gm. per kilo) were introduced; the amount eliminated by the kidneys was uncertain; no bile pigment appeared in the urine. A second injection of 3.08 gm. of hemoglobin (0.32 gm. per kilo) was made one day later. The amount eliminated by the kidneys was 0.65 gm., and the amount retained was 2.43 gm. (0.25 gm. per kilo). Bile pigment was present in the urine. The amount of hemoglobin eliminated by the kidneys was 21.1 per cent.

Experiment XIII.—July 8, 1911. Splenectomy.

April 2, 1912. (Nine months after splenectomy.) Weight 17,220 gm. Ether anesthesia; needle in small vein of leg. Urine contained no albumin and no bile.

3.17–3.27 P. M. Injected 167.5 c.c. of solution containing 6.57 gm. of hemoglobin.

April 3, 1912. 8.00 A. M. The cage urine contained 1.81 gm. of hemoglobin and had a faint trace of bile.

April 4, 1912. Urine contained no hemoglobin, no albumin, and no bile.

Summary.—The amount of hemoglobin injected was 6.57 gm. (0.38 gm. per kilo), the amount eliminated by the kidneys was 1.81 gm., and the amount retained was 4.76 gm. (0.28 gm. per kilo). Bile pigments were present in the urine in traces. The hemoglobin eliminated by the kidneys equalled 27.5 per cent. of the total amount injected.

Experiment XIV.—February 10, 1912. Splenectomy.

April 11, 1912. Weight of animal 7,520 gm. (Sixty-one days after splenectomy.) Ether anesthesia; needle in small vein of leg.

Urine contained no albumin and no bile.

3.33–3.38 P. M. Injected 59.8 c.c. of a solution containing 2.09 gm. of hemoglobin.

7.20 P. M. The cage urine contained 0.15 gm. of hemoglobin, no albumin, and no bile.

April 12, 1912. 8.00 A. M. The cage urine was free from hemoglobin; bile pigment present; no albumin.

9.30 A. M. Urine obtained by catheter showed distinct reaction for bile.

April 13, 1912. No bile.

Summary.—The amount of hemoglobin injected was 2.09 gm. (0.28 gm. per kilo), the amount eliminated by the kidneys was 0.15 gm., and the amount retained was 1.94 gm. (0.26 gm. per kilo). Bile pigments were present in the urine in considerable amount. The hemoglobin eliminated by the kidney equalled 7.2 per cent. of the total amount injected.

Experiment XV.—February 10, 1912. Splenectomy.

April 10, 1912. Weight 12,680 gm. (Sixty days after splenectomy.) Ether anesthesia; needle in small vein of leg.

No albumin and no bile in urine.

4.22–4.29 P. M. Injected 99 c.c. of solution containing 3.46 gm. of hemoglobin.

April 11, 1912. 8.45 A. M. The cage urine contained 0.36 gm. of hemoglobin in solution and about 0.28 gm. of precipitated hemoglobin; the total amount eliminated was about 0.64 gm. No albumin; faint trace of bile.

9.30 A. M. Urine obtained by catheter contained a trace of bile; no hemoglobin.

April 12, 1912. No bile.

Summary.—The amount of hemoglobin injected equalled 3.46 gm. (0.27 gm. per kilo), the amount eliminated by the kidneys was about 0.64 gm., and the amount retained was about 2.82 gm. (0.22 gm. per kilo). A trace of bile appeared in the urine. The amount of hemoglobin eliminated by the kidneys was about 18.4 per cent. of the total amount injected.

These six experiments on splenectomized animals, in all of which bile pigments appeared in the urine for a short time and in small quantities after the retention of 0.44, 0.31, 0.25, 0.28, 0.26, and 0.22 gm. per kilo, respectively, indicate that the threshold for jaundice in splenectomized dogs is approximately 0.22 of a gram per kilo, the same as in the experiments (VI to XI) with normal dogs, in which the threshold was found to be between 0.18 and 0.22 of a gram per kilo.

When we examine the percentage of hemoglobin eliminated by the kidneys in the six splenectomized animals, we find that it runs a trifle lower than the limits determined for normal animals, being 18.8, 16.8, 21.1, 27.5, 7.2 (?), and 18.4 per cent. (average, excluding the fifth figure, 20.5 per cent.), as compared with 32.5, 26.8, 17.1, 35.9, 16, and 26.5 per cent. (experiments VI to XI), with an average of 25.8 per cent. We can, therefore, conclude that splen-

ectomy has no influence in increasing the elimination of free hemoglobin by the kidneys nor does it, as is shown by the occurrence of choluria in each of the experiments, alter the ability of the liver to form bile pigments from hemoglobin, or interfere with the elimination of these pigments. Thus one of the possible explanations for the failure of jaundice to follow the administration of a hemolytic serum in splenectomized animals, as suggested in the first paper[2] of this series, is shown to be untenable.

Our next experiments were undertaken for the purpose of determining whether a liver saturated with bile pigments would take up hemoglobin from the serum less rapidly than a normal liver and thus lead to the elimination of a larger percentage of hemoglobin through the kidneys. For this purpose we used (1) a dog that, after splenectomy, had developed a spontaneous choluria, and (2) a dog with obstructive jaundice. The observations on the former follow.

Experiment XVI.—November 15, 1911. Splenectomy.

March 4, 1912. Weight of animal 15,550 gm. (110 days after splenectomy). Ether anesthesia; needle in small vein of leg.

Urine intensely stained with bile pigment.

Injection during a period of ten minutes of 104 c.c. of a solution containing 3.92 gm. of hemoglobin.

March 5, 1912. 8 A. M. The cage urine contained 1.12 gm. of hemoglobin. Urine was deeply bile-stained.

5.00 P. M. The cage urine was free from hemoglobin, but bile pigment was still abundant.

Summary.—The amount of hemoglobin injected equalled 3.92 gm. (0.25 gm. per kilo), the amount eliminated by the kidneys was 1.12 gm., and the amount retained was 2.80 gm. (0.18 gm. per kilo). Bile pigments continued to be as abundant as before the experiment. The hemoglobin eliminated by the kidneys equalled 28.6 per cent. of the total amount injected.

The second experiment was on a splenectomized dog in which we ligated the common bile duct two days before the injection of the hemoglobin.

Experiment XVII.—Female dog.

March 6, 1912. Splenectomy.

March 27, 1912. Common bile duct doubly ligated. Severed between ligatures.

March 28, 1912. Large amount of bile in urine.

[2] Pearce, R. M., Austin, J. H., and Krumbhaar, E. B., I. Reactions to Hemolytic Serum at Various Intervals after Splenectomy, *Jour. Exper. Med.*, 1912, xvi, 363.

March 29, 1912. Much bile and no albumin in urine. Weight 8,945 gm.

3.17–3.24 P. M. Ether anesthesia; needle in small vein of leg. Injected 90.5 c.c. of solution containing 2.23 gm. of hemoglobin.

11.50 P. M. The cage urine contained 0.59 gm. of hemoglobin; trace of albumin; intense bile reaction.

March 30, 1912. The cage urine contained no hemoglobin; trace of albumin; intense bile reaction.

March 31, 1912. Urine the same as on March 30.

Summary.—The amount of hemoglobin injected equalled 2.23 gm. (0.25 gm. per kilo), the amount eliminated was 0.59 gm., and the amount retained was 1.67 gm. (0.19 gm. per kilo). Bile pigments continued to be abundant in the urine. The hemoglobin eliminated by the kidneys equalled 26.4 per cent. of the total amount injected.

The percentage of hemoglobin eliminated by the kidney in these two experiments, namely 28.6 and 26.4 per cent., is distinctly higher than in the six other experiments on splenectomized dogs (with the single exception of experiment XIII), although well within the limits established for normal dogs. With the increased elimination, there was a corresponding slight decrease in the amount retained, as compared with both normal and splenectomized dogs. We conclude, therefore, that saturation of the liver with bile pigments may diminish, but only to a very slight extent, the relative amount of hemoglobin removed from the serum by the liver with a concomitant very slight increase in the amount removed by the kidneys.

SUMMARY.

The results of this study may be stated as follows.

1. Rapid injection of more than 0.06 of a gram per kilo of hemoglobin intravenously into a normal animal is followed by the appearance of hemoglobin in the urine (pelvis of kidney) within eight to ten minutes.

2. After rapid injection of more than 0.012 of a gram per kilo per minute of hemoglobin, 16 to 36 per cent. of the total amount, if this equals 0.25 of a gram per kilo, is eliminated in the urine and is accompanied by choluria.

3. If the injection of not more than 0.35 of a gram per kilo is made slowly (less than 0.01 of a gram per kilo per minute), the amount eliminated in the urine is only 2.33 to 9.5 per cent. of the total amount injected, and choluria does not occur.

4. The concentration of free hemoglobin in the blood which constitutes the threshold value of the kidneys for hemoglobin is approximately 0.06 of a gram of hemoglobin per kilo of body weight. When about this concentration is reached, hemoglobin appears in the urine.

5. The amount of hemoglobin per kilo of body weight which, after rapid injection, may be retained without jaundice, is approximately 0.18 of a gram. When 0.22 or 0.23 of a gram is retained bile pigments appear in the urine. The threshold of the liver for jaundice in point of hemoglobin saturation lies, therefore, between 0.18 and 0.22 of a gram per kilo of body weight. With slow injections a greater amount may be retained without choluria.

6. The absence of the spleen does not alter greatly the percentage of hemoglobin eliminated by the kidney, nor does it raise the threshold of the liver for jaundice.

7. In the presence of jaundice, either hemolytic or obstructive, the amount of hemoglobin retained by splenectomized animals is slightly diminished and that eliminated by the kidneys is correspondingly increased.

Upon these data may be based the following explanation of the mechanism by which free hemoglobin is removed from the blood serum. Hemoglobin is not removed by the kidney until its concentration in the blood serum reaches a certain level (0.06 of a gram of free hemoglobin per kilo of body weight). This constitutes the threshold value of the kidneys for hemoglobin and when it is reached hemoglobin appears in the urine. When the concentration is lower, hemoglobinuria ceases; at the same time, however, the liver, and possibly other tissues, take up hemoglobin as soon as mere traces are present in the serum and they continue this removal whether the renal threshold is exceeded or not. The two processes go on simultaneously, the rate of removal, when the renal threshold is exceeded, being for the kidneys 17 to 36 per cent., and for the liver and other tissues 64 to 83 per cent, of the total amount introduced. The hemoglobin which is removed by the liver is transformed into bile pigments. If the amount reaching the liver is small and is received slowly, the amount of bile formed is not increased above the excretory capacity of the liver, and it is removed by the bile passages

without the occurrence of choluria. This is shown in our experiments in which injections of hemoglobin were made more slowly than 0.01 of a gram per kilo per minute. On the other hand, if the hemoglobin is taken up by the liver rapidly and in large amounts, the bile capillaries are overtaxed and the bile cannot be rapidly removed, but is reabsorbed into the blood, and choluria develops.

If this theory is correct we have an explanation of those instances of blood destruction in man characterized by jaundice, but not accompanied by hemoglobinuria. In a slow, gradual destruction of the red blood cells, the liver removes the hemoglobin from the serum so rapidly that the concentration of hemoglobin in the serum does not reach the threshold value of the kidneys and hemoglobinuria, therefore, cannot occur. The constant absorption of large amounts of hemoglobin by the liver and the increase in bile formation which results does, however, overtax the bile passages and jaundice occurs.

In the same way may be explained the continuance of jaundice after the disappearance of a transient hemoglobinuria. A rapid destruction of a large amount of blood raises the concentration of hemoglobin in the serum so quickly that the threshold value of the kidney is quickly exceeded and hemoglobin appears in large amounts in the urine. When an amount of hemoglobin sufficient to reduce the concentration of the serum below the threshold value of the kidney has been removed, a considerable amount of hemoglobin may still remain in the serum, and it is the slow elimination of this through the liver that causes the choluria to continue.

The demonstration that the absence of the spleen has no important influence on the elimination of hemoglobin by the kidney, on its transformation into bile pigments, or on the removal of such pigments, is of interest in connection with an observation made in the first paper of this series. This was concerning the frequent failure of jaundice to follow the administration of hemolytic serum during the early period following splenectomy.[3] Among the possible explanations was the suggestion that the spleen is in some way concerned in the disintegration of free hemoglobin or in the elaboration of its derivatives. The present investigations demonstrate that such an explanation is without experimental basis, though it does not

[3] Pearce, R. M., Austin, J. H., and Krumbhaar, E. B., *loc. cit.*

controvert the possibility of the spleen being concerned in liberating hemoglobin from the red cells and suggests that the failure of jaundice is due to some other factor or factors. Evidence to indicate that the changes in the blood that follow splenectomy are important factors is offered in the third paper[4] of this series.

[4] Pearce, R. M., Austin, J. H., and Musser, J. H., Jr., III. The Changes in the Blood Following Splenectomy and Their Relation to the Production of Hemolytic Jaundice, *Jour. Exper. Med.*, 1912, xvi, in press.